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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
)
BEN SHEN, WEN LIU, STEVEN D.)
CHRISTENSON and SCOTT STANDAGE)
)
For: GENE CLUSTER FOR)
PRODUCTION OF THE ENEDIYNE)
ANTITUMOR ANTIBIOTIC C-1027)
) San Francisco, California

Patent Application
Assistant Commissioner for Patents
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PATENT APPLICATION TRANSMITTAL

Sir:

Transmitted herewith for filing is the patent application of inventor(s) Ben Shen, Wen Liu, Steven D. Christenson and Scott Standage, for "GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTIC C-1027." Enclosed are:

1. 64 pages of the specification, including 71 claims and an abstract.
2. 11 sheets of drawings.
3. 79 pages of Sequence Listing.
4. An oath or declaration of the inventors (unsigned).

The filing fee is being deferred at this time.

Dated: January 5, 2000.

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U.S. Patent Application For

GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTIC C-1027

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GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTIC C-1027

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit under 35 U.S.C. §119 of provisional application USSN 60/115,434, filed on January 6, 1999, which is herein incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This work was supported in part by a grant from the Cancer Research Coordinating Committee, University of California, the National Institutes of Health grant CA78747, and the Searle Scholars Program/The Chicago Community Trust. The Government of the United States of America may have certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the field of enediyne antibiotics. In particular this invention elucidates the gene cluster controlling the biosynthesis of the C-1027 enediyne.

BACKGROUND OF THE INVENTION

The enediyne antibiotics are currently the focus of intense research activity in the fields of chemistry, biology, and medical sciences, because of their unique molecular architecture, biological activities, and modes of actions (Doyle and Borders (1995) *Enediyne antibiotics as antitumor agents.* Marcel-Dekker, New York, Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188). Since the unveiling of the structure of neocarzinostatin chromophore (Edo *et al.* (1985) *Tetrahedron Lett.* 26: 331-340) in 1985, the enediyne family has grown steadily. Thus far, there have been three basic groups within the enediyne antibiotic family: (a) the calicheamicin/esperamicin type, which includes the calicheamicins, the esperamicins, and namenamicin, (b) the dynemicin type, and (c) the chromoprotein type, consisting of an apoprotein and an unstable enediyne chromophore. The latter group includes neocarzinostatin, kedarcidin, C-1027 (Fig. 1), and maduropeptin, whose enediyne chromophore structures have been established, as well as several others whose enediyne chromophore structures are yet to be determined due to their instability (Thorson *et al.*

(1999) *Bioorg. Chem.*, 27: 172-188). N1999A2, in contrast to the other chromoproteins, exists as an enediyne chromophore alone despite the fact that its structure is very similar to the other chromoprotein chromophore (*Ando et al.*(1998) *Tetra. Letts.*, 39: 6495-6480).

As a family, the enediyne antibiotics are the most potent, highly active antitumor agents ever discovered. Some members are 1000 times more potent than adriamycin, one of the most effective, clinically used antitumor antibiotics (Zhen *et al.* (1989) *J. Antibiot.* 42: 1294-1298). All members of this family contain a unit consisting of two acetylenic groups conjugated to a double bond or incipient double bond within a nine or ten-membered ring; i.e., the enediyne core as exemplified by C-1027 in Fig. 1. As the consequence of this structural feature, these compounds share a common mechanism of action: the enediyne core undergoes an electronic rearrangement to form a transient benzenoid diradical, which is positioned in the minor groove of DNA so as to damage DNA by abstracting hydrogen atoms from deoxyriboses on both strands (Fig. 1). Reaction of the resulting deoxyribose carbon-centered radicals with molecular oxygen initiates a process that results in both single-strand and double-strand DNA cleavages (Doyle and Borders (1995) *Enediyne antibiotics as antitumor agents.* Marcel-Dekker, New York; Ikemoton *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:10506-10510; Myers *et al.* (1997) *J. Am. Chem. Soc.* 119: 2965-2972; Stassinopoulos *et al.* (1996) *Science* 272: 1943-1946; Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188; Xu *et al.* (1997) *J. Am. Chem. Soc.* 119: 1133-1134). This novel mechanism of DNA damage has important implications for their application as potent cancer chemotherapeutic agents (Doyle and Borders (1995) *supra.*; Sievers *et al.* (1999) *Blood* 93: 3678-3684).

As an alternative to making structural analogs of microbial metabolites by chemical synthesis, manipulations of genes governing secondary metabolism offer a promising alternative allowing preparation of these compounds biosynthetically (Cane *et al.* (1998) *Science* 282: 63-68; Hutchinson and Fujii. (1995) *Ann. Rev. Microbiol.* 49: 201-38; Katz and Donadio (1993) *Ann. Rev. Microbiol.* 47: 875-912). The success of the latter approach depends critically on the availability of novel genetic systems and on genes encoding novel enzyme activities. The enediynes offer a distinct opportunity to study the biosynthesis of their unique molecular scaffolds and the mechanism of self-resistance to extremely cytotoxic natural products. Elucidation of these aspects provides access to rational engineering of enediyne biosynthesis for novel drug leads and makes it possible to construct enediyne overproducing strains by de-regulating the biosynthetic machinery. In

addition, elucidation of an enediyne gene cluster contributes to the general field of combinatorial biosynthesis by expanding the repertoire of novel polyketide synthase (PKS) and deoxysugar biosynthesis genes as well as other genes uniquely associated with enediyne biosynthesis, leading to the making of novel enediynes via combinatorial biosynthesis.

SUMMARY OF THE INVENTION

This invention provides nucleic acid sequences and characterization of the gene cluster responsible for the biosynthesis of the enediyne C-1027 (produced by *Streptomyces globisporus*). In particular structural and functional characterization is provided for the 50 open reading frames (ORFs) comprising this gene cluster. Thus, in one embodiment, this invention provides an isolated nucleic acid comprising a nucleic acid selected from the group consisting of a nucleic acid encoding any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA), a nucleic acid encoding a polypeptide encoded by any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA); and a nucleic acid amplified by polymerase chain reaction (PCR) using primer pairs that amplify any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA). In one embodiment, preferred nucleic acids comprise a nucleic acid encoding at least two (more preferably at least three or more) open reading frames (ORFs) selected from the group consisting of ORF-1 through ORF 42, excluding ORF 9 (cagA).

In another embodiment this invention provides an isolated nucleic acid comprising a nucleic acid that specifically hybridizes under stringent conditions to an open reading frame (ORF) of the C-1027 biosynthesis gene cluster, excluding ORF 9 (cagA), and can substitute for the ORF to which it specifically hybridizes to direct the synthesis of an enediyne. In certain embodiments this also includes nucleic acids that would stringently hybridizes indicated above, but for, the degeneracy of the nucleic acid code. In other words, if silent mutations could be made in the subject sequence so that it hybridizes to he indicated sequence(s) under stringent conditions, it would be included in certain embodiments. Particularly preferred nucleic acids comprises a nucleic acid that specifically hybridizes under stringent conditions to a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, ORF 14, ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF

26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42. Particularly preferred isolated nucleic acid comprises a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, ORF 14, ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42. The nucleic acid may comprises a nucleic acid that is a single nucleotide polymorphism (SNP) of a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 81, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, ORF 14, ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42.

This invention also provides an isolated gene cluster comprising open reading frames encoding polypeptides sufficient to direct the assembly of a C-1027 enediyne or a C-1027 enediyne analogue. The gene cluster may be present in a cell, more preferably in a bacterial cell (*e.g. Actinomycetes, Actinoplanetes, Actinomadura, Micromonospora,* or *Streptomycetes*). Particular preferred bacterial cells include, but are not limited to *Streptomyces globisporus, Streptomyces lividans, Streptomyces coelicolor, Micromonospora echinospora spp. calichenisis, Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus*, and *Actinomycete* L585-6. The gene cluster may contain one or more open reading frames is operatively linked to a heterologous promoter (*e.g.* a constitutive or an inducible promoter).

This invention also provides for an polypeptide encoded by any one or more of the nucleic acids described herein.

Also provided are host cell(s) (*e.g.* eukaryotic cells or bacterial cells as described herein) transformed with one or more of the expression vectors described herein. Preferred host cells are transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the assembly of a C-1027 enediyne or a C-1027 enediyne analogue. In certain embodiments, heterologous nucleic acid may comprise only a portion of the gene cluster, but the cell will still be able to express an enediyne.

This invention also provides methods of chemically modifying a biological molecule. The methods involve contacting a biological molecule that is a substrate for a polypeptide encoded by a C-1027 biosynthesis gene cluster open reading frame, with a polypeptide encoded by a C-1027 biosynthesis gene cluster open reading frame whereby the polypeptide chemically modifies the biological molecule. In one preferred embodiment, the polypeptide is an enzyme selected from the group consisting of a hydroxylase, a homocysteine synthase, a dNDP-glucose dehydrogenase, a citrate carrier protein, a C-methyl transferase, an N-methyl transferase, an aminotransferase, a CagA apoprotein, an NDP-glucose synthase, an epimerase, an acyl transferase, a coenzyme F390 synthase, and epoxidase hydrolase, an anthranilate synthase, a glycosyl transferase, a monooxygenase, a type II condensation protein, an aminomutase, a type II adenylation protein, an O-methyl transferase, a P-450 hydroxylase, an oxidoreductase, and a proline oxidase. In a preferred embodiment the method involves contacting the biological molecule with at least two (preferably at least three or more) different polypeptides encoded by C-1027 biosynthesis gene cluster open reading frames. The contacting may be in a host cell (*e.g.* a eukaryotic cell or a bacterial cell) or the contacting can be *ex vivo*. The biological molecule can be an endogenous metabolite produced by said host cell or an exogenous supplied metabolite. In preferred embodiments, the host cell is a bacterial cell or eukaryotic cell (*e.g.*, a mammalian cell, a yeast cell, a plant cell, a fungal cell, an insect cell, *etc.*). In certain preferred embodiments, the host cell synthesizes sugars and glycosylates the biological molecule. In other preferred embodiments, the host cell synthesizes deoxysugars. The method can further involve contacting the biological molecule with a polyketide synthase or a non-ribosomal polypeptide synthetase. The contacting can be in a cell (*e.g.*, a bacterial cell) or *ex vivo*. In one preferred embodiment the method comprises contacting the biological molecule with at substantially all of the polypeptides encoded by C-1027 biosynthesis gene cluster open reading frames and said method produces an enediyne or enediyne analogue. In another preferred embodiment, the biological molecule is a fatty acid and the biological molecule is contacted with a C-1027 orf polyeptide selected from the group consisting of an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase. In certain embodiments, the biological molecule is a fatty acid and said biological molecule is contacted with a plurality of C-1027 orf polypeptides comprising an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase. In one especially preferred

embodiment ,the biological molecule is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38. In another especially preferred embodiment, the biological molecule is contacted with polypeptides encoded by ORF 15, ORF 16, ORF 28, ORF3, ORF 14, and ORF 13, and, in certain embodiments, ORF 4 and ORF 3 as well.

In certain embodiments, the method may comprise contacting a sugar with one or more C-1027 open reading frame polypeptides selected from the group consisting of a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase. Particularly preferred variant of this method comprise contacting a dNDP-glucose with a plurality of C-1027 open reading frame polypeptides comprising a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase.

In certain other embodiments, the method comprises contacting an amino acid with one or one or more C-1027 open reading frame polypeptides selected from the group consisting of a hydroxylase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein. These methods may involve contacting an amino acid with a plurality of C-1027 open reading frame polypeptides comprising a hydroxylase, a halogenase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein. In particularly preferred embodiments, the amino acid is a tyrosine.

This invention also provides a method of synthesizing a chromaprotein type enediyne core, said method comprising contacting a fatty acid with one or more C-1027 orf polypeptides selected from the group consisting of an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase. In preferred embodiments, the fatty acid may be contacted with a plurality of C-1027 orf polypeptides comprising an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase. In particularly preferred embodiments, the fatty acid is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38.

In still yet another embodiment, this invention provides a method of synthesizing a deoxysugar. This method involves contacting a sugar with one or more C-1027 open reading frame polypeptides selected from the group consisting of a dNDP-glucose

synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase. In preferred embodiments, this method involves contacting a dNDP-glucose with a plurality of C-1027 open reading frame polypeptides comprising a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase. In particularly preferred embodiments, the dNDP-glucose is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38.

This invention also provides methods of synthesizing a beta amino acid by contacting an amino acid with one or one or more C-1027 open reading frame polypeptides selected from the group consisting of a hydroxylase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein. The method preferably comprises contacting an amino acid with a plurality of C-1027 open reading frame polypeptides comprising a hydroxylase, a halogenase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein. Particularly preferred embodiments comprise contacting the amino acid (*e.g.* tyrosine) with polypeptides encoded by ORF 4, ORF11, ORF24, ORF23, ORF25, and ORF26.

Also provided are methods of synthesizing an enediyne or an enediyne analogue. These methods involve culturing a cell (*e.g.* a eukaryotic cell or a bacterium) comprising a recombinantly modified C-1027 gene cluster under conditions whereby said cell expresses said enediyne or enediyne analogue; and recovering the enediyne or enediyne analogue. In preferred embodiments, the gene cluster is present in a bacterium (*e.g., Actinomycetes, Actinoplanetes, Actinomadura, Micromonospora*, or *Streptomycetes*). Particularly preferred bacteria include, but are not limited to *Streptomyces globisporus, Streptomyces lividans, Streptomyces coelicolor, Micromonospora echinospora spp. calichenisis, Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus,* and *Actinomycete* L585-6. In another preferred embodiment, the gene cluster is present in a eukaryotic cell (*e.g.* a mammalian cell, a yeast cell, a plant cell, a fungal cell, an insect cell, *etc.*). The host cell can be one that synthesizes sugars and glycosylates the enediyne or enediyne analogue. The host can be one that synthesizes deoxysugars.

This invention also provides a method of making a cell (*e.g.,* a bacterial or eukaryotic cell) resistant to an enediyne or an enediyne metabolite. This method involves expressing in the cell one or more isolated C-1027 open reading frame nucleic acids that encode a protein selected from the group consisting of a CagA apoprotein, a SgcB transmembrane efflux protein, a transmembrane transport protein, a Na+/H+ transporter, an ABC transport, a glycerol phosphate tranporter, and a UvrA-like protein. In preferred embodiments, the isolated C-1027 open reading frame nucleic acids are selected from the group consisting of ORF 9, ORF2, ORF 27, ORF 0, ORF 1 c-terminus, ORF 2, and ORF 1 N-terminus. Certain embodiments exclude cagA (ORF 9).

In one embodiment, this invention specifically excludes one or more of open reading frames -7 through 42. In particular, in one embodiment this invention excludes cagA (ORF 9), and/or sgcA (ORF 1), and/or sgcB (ORF 2).

DEFINITIONS

The terms "C-1027 open reading frame", and "C-1027 ORF" refer to an open reading frame in the C-1027 biosynthesis gene cluster as isolated from *Streptomyces globisporus.* The term also embraces the same open reading frames as present in other enediyne-synthesizing organisms (*e.g.* other strains and/or species of *Streptomyces, Actinomyces,* and the like). The term encompasses allelic variants and single nucleotide polymorphisms (SNPs). In certain instances the C-1027 ORF is used synonymously with the polypeptide encoded by the C-1027 ORF and may include conservative substitutions in that polypeptide. The particular usage will be clear from context.

The terms "isolated" "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. With respect to nucleic acids and/or polypeptides the term can refer to nucleic acids or polypeptides that are no longer flanked by the sequences typically flanking them in nature.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The term also includes variants on the traditional peptide linkage joining the amino acids making up the polypeptide.

The terms "nucleic acid" or "oligonucleotide" or grammatical equivalents herein refer to at least two nucleotides covalently linked together. A nucleic acid of the present invention is preferably single-stranded or double stranded and will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage *et al.* (1993) *Tetrahedron* 49:1925) and references therein; Letsinger (1970) *J. Org. Chem.* 35:3800; Sprinzl et al. (1977) *Eur. J. Biochem.* 81: 579; Letsinger *et al.* (1986) *Nucl. Acids Res.* 14: 3487; Sawai *et al.* (1984) *Chem. Lett.* 805, Letsinger *et al.* (1988) *J. Am. Chem. Soc.* 110: 4470; and Pauwels *et al.* (1986) *Chemica Scripta* 26: 141 9), phosphorothioate (Mag et al. (1991) *Nucleic Acids Res.* 19:1437; and U.S. Patent No. 5,644,048), phosphorodithioate (Briu *et al.* (1989) *J. Am. Chem. Soc.* 111 :2321, O-methylphophoroamidite linkages (*see* Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (*see* Egholm (1992) *J. Am. Chem. Soc.* 114:1895; Meier *et al.* (1992) *Chem. Int. Ed. Engl.* 31: 1008; Nielsen (1993) *Nature*, 365: 566; Carlsson *et al.* (1996) *Nature* 380: 207). Other analog nucleic acids include those with positive backbones (Denpcy *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 6097; non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Angew. (1991) *Chem. Intl. Ed. English* 30: 423; Letsinger et al. (1988) *J. Am. Chem. Soc.* 110:4470; Letsinger *et al.* (1994) *Nucleoside & Nucleotide* 13:1597; Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker *et al.* (1994), *Bioorganic & Medicinal Chem. Lett.* 4: 395; Jeffs *et al.* (1994) *J. Biomolecular NMR* 34:17; *Tetrahedron Lett.* 37:743 (1996) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, *Carbohydrate Modifications in Antisense Research*, Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (*see* Jenkins *et al.* (1995), *Chem. Soc. Rev.* pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally associated with a

region of a recombinant construct, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

A "coding sequence" or a sequence which "encodes" a particular polypeptide (*e.g.* a PKS, an NRPS, *etc.*), is a nucleic acid sequence which is ultimately transcribed and/or translated into that polypeptide *in vitro* and/or *in vivo* when placed under the control of appropriate regulatory sequences. In certain embodiments, the boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eucaryotic mRNA, genomic DNA sequences from procaryotic or eucaryotic DNA, and even synthetic DNA sequences. In preferred embodiments, a transcription termination sequence will usually be located 3' to the coding sequence.

Expression "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

"Recombination" refers to the reassortment of sections of DNA or RNA sequences between two DNA or RNA molecules. "Homologous recombination" occurs between two DNA molecules which hybridize by virtue of homologous or complementary nucleotide sequences present in each DNA molecule.

The terms "stringent conditions" or "hybridization under stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid

hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2 Overview of principles of hybridization and the strategy of nucleic acid probe assays*, Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see*, Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual (2nd ed.)* Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.,* when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Expression vectors are defined herein as nucleic acid sequences that are direct the transcription of cloned copies of genes/cDNAs and/or the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes or cDNAs in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Expression vectors include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Specifically designed vectors allow the shuttling of DNA

between hosts, such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector preferably contains: an origin of replication for autonomous replication in a host cell, a selectable marker, optionally one or more restriction enzyme sites, optionally one or more constitutive or inducible promoters. In preferred embodiments, an expression vector is a replicable DNA construct in which a DNA sequence encoding a one or more PKS and/or NRPS domains and/or modules is operably linked to suitable control sequences capable of effecting the expression of the products of these synthase and/or synthetases in a suitable host. Control sequences include a transcriptional promoter, an optional operator sequence to control transcription and sequences which control the termination of transcription and translation, and so forth.

The term "conservative substitution" is used in reference to proteins or peptides to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (e.g. charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

The "group consisting of ORF-1 through ORF 42" refers to the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 81, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 9, ORF 10, ORF 11, ORF 12, ORF 13, ORF 14, ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42 as identified in Tables I and II. In certain embodiments ORF 9 (cagA) is excluded.

A "biological molecule that is a substrate for a polypeptide encoded by a enediyne (*e.g.,* C-1027) biosynthesis gene" refers to a molecule that is chemically modified by one or more polypeptides encoded by open reading frame(s) of the C-1027 biosynthesis gene cluster. The "substrate" may be a native molecule that typically participates in the biosynthesis of an enediyne, or can be any other molecule that can be similarly acted upon by the polypeptide.

A "polymorphism" is a variation in the DNA sequence of some members of a species. A polymorphism is thus said to be "allelic," in that, due to the existence of the polymorphism, some members of a species may have the unmutated sequence (*i.e.* the original "allele") whereas other members may have a mutated sequence (*i.e.* the variant or mutant "allele"). In the simplest case, only one mutated sequence may exist, and the polymorphism is said to be diallelic. In the case of diallelic diploid organisms, three genotypes are possible. They can be homozygous for one allele, homozygous for the other allele or heterozygous. In the case of diallelic haploid organisms, they can have one allele or the other, thus only two genotypes are possible. The occurrence of alternative mutations can give rise to trialleleic, *etc.* polymorphisms. An allele may be referred to by the nucleotide(s) that comprise the mutation.

"Single nucleotide polymorphism" or "SNPs are defined by their characteristic attributes. A central attribute of such a polymorphism is that it contains a polymorphic site, "X," most preferably occupied by a single nucleotide, which is the site of the polymorphism's variation (Goelet and Knapp U.S. patent application Ser. No. 08/145,145). Methods of identifying SNPs are well known to those of skill in the art (*see, e.g.,* U.S. Patent 5,952,174).

Abbreviations used herein include LB, Luria-Bertani; NGDH, dNDP-glucose 4,6-dehydratase ; nt, nucleotide; ORF, open reading frame; PCR, polymerase chain reaction; PEG, polyethyleneglycol; PKS, polyketide synthase; RBS, ribosomal binding site; Apr, apramycin; R, resistant; Th, thiostrepton; WT, wild-type; and TS, temperature sensitive

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the structures of C-1027 chromophore and the benzenoid diradical intermediate proposed to initiate DNA cleavage.

Figure 2 illustrates a scheme using C-1027 open reading frame polypeptides for the synthesis of deoxysugars.

Figure 3A illustrates a scheme using C-1027 open reading frame polypeptides for the synthesis of a β-amino acid.

Figure 3B illustrates a scheme using C-1027 open reading frame polypeptides for the synthesis of a benzoxazolinate.

Figure 4 illustrates the synthesis of the enediyne core and final assembly of the C-1027 enediyne.

Figures 5A, 5B, and 5C illustrate the organization of the C-1027 enediyne biosynthetic gene cluster. Figure 5A shows a restriction map of the 75-kb *sgc* gene cluster from *S. globisporus* as represented by three cosmid clones. Figure 5B illustrates the genetic organization of the *sgcA, sgcB*, and *cagA* genes, showing that they are clustered in the *sgc* gene cluster. Probe 1, the 0.55-kb dNDP-glucose 4,6-dehydratase gene fragment from pBS1002. Probe 2, the 0.73-kb *cagA* fragment from pBS1003. A, *Apa*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I, S, *Sac*II; Sp, *Sph*I. Figure 5C shows the genetic organization of the C-1027 biosynthesis gene cluster.

Figure 6 shows the DNA and deduced amino acid sequences of the 3.0-kb *Bam*HI fragment from pBS1007, showing the *sgcA* and *sgcB* genes. Possible RBSs are boxed. The presumed translational start and stop sites are in boldface. Restriction enzyme sites of interest are underlined. The amino acids, according to which the degenerated PCR primer were designed for amplifying the dNDP-glucose 4,6-dehydratase gene from *S. globisporus*, are underlined.

Figure 7 shows the amino acid sequence alignment of SgcA with three other dNDP-glucose 4,6-dehydratases. Gdh, TDP-glucose 4,6-dehydratase of *S. erythraea* (AAA68211); MtmE, TDP-glucose 4,6-dehydratase in the mithramycin pathway of *S. argillaceus* (CAA71847); TylA2, TDP-glucose 4,6-dehydratase in the tylosin pathway of *S. fradiae* (S49054). Given in parentheses are protein accession numbers. The αβα fold with the NAD^+-binding motif of GxGxxG is boxed.

Figures 8A and 8B show disruption of *sgcA* by single crossover homologous recombination. Figure 8A shows construction of *sgcA* disruption mutant and restriction maps of the wild-type *S. globisporus* C-1027 and *S. globisporus* SB1001 mutant strains showing predicted fragment sizes upon *Bam*HI digestion. Figures 8B and 8C show a Southern analysis of *S. globisporus* C-1027 (lane 1) and *S. globisporus* SB1001 (lanes 2, 3, and 4, three individual isolates) genomic DNA, digested with *Bam*HI, using (Figure 8B) pOJ260 vector or (Figure 8C) the 0.75-kb *Sac*II/*Kpn*I fragment of *sgcA* from pBS1012 as a probe, respectively. B, *Bam*HI; K, *Kpn*I; S, *Sac*II.

Figures 9A, 9B, 9B, and 9D illustrate the determination of C-1027 production in various *S. globisporus* strains by assaying their antibacterial activity against *M. luteus*.

Figure 9A:1, *S. globisporus*C-1027; 2,3, and 4, *S. globisporus* SB1001 (three individual isolates); 5, *S. globisporus* AF67; 6, *S. globisporus* AF40. Figure 9B: 1, *S. globisporus* C-1027; 2, *S. globisporus* SB1001 (pWHM3); 3 and 4, *S. globisporus* SB1001 (pBS1015) (two individual isolates). Both *S. globisporus* SB1001 (pWHM3) and *S. globisporus* SB1001 (pBS1015) were grown in the presence of 5 μg/mL thiostrepton. Figure 9C: 1, *S. globisporus*C-1027; 2, *S. globisporus* SB1001 (pBS1015); 3. *S. globisporus* SB1001; 4. *S. globisporus* SB1001 (pWHM3); 5. *S. globisporus* AF40; 6. *S. globisporus* AF44. All *S. globisporus* strains were grown in the absence of thiostrepton. Figure 9D: 1. *S. globisporus* (pKC1139); 2. *S. globisporus* (pBS1018).

DETAILED DESCRIPTION

This invention provides a complete gene cluster regulating the biosynthesis of C-1027, the most potent member of the enediyne antitumor antibiotic family. C-1027 is produced by *Streptomyces globisporus* C-1027 and consists of an apoprotein (encoded by the *cagA* gene) and a non-peptidic chromophore. The C-1027 chromophore could be viewed as being derived biosynthetically from a benzoxazolinate, a deoxyamino hexose, a β-amino acid, and an enediyne core. Adopting a strategy to clone the C-1027 biosynthesis gene cluster by mapping a putative dNDP-glucose 4,6-dehydratase (NGDH) gene to *cagA*, we localized 75 kb contiguous DNA from *S. globisporus* encoding a complete C-1027 gene cluster.

Initial sequencing of the cloned gene cluster revealed two genes, *sgcA* and *sgcB,* that encode an NGDH enzyme and a transmembrane efflux protein, respectively, and confirmed that the *cagA* gene resides approximately 14 kb upstream of the *sgcA,B* locus. The involvement of the cloned gene cluster in C-1027 biosynthesis was demonstrated by disrupting the *sgcA* gene to generate C-1027-nonproducing mutants and by complementing the *sgcA* mutants in vivo to restore C-1027 production.

Subsequent DNA sequence analysis provided the complete enediyne C-1027 gene cluster sequence (SEQ ID NOs: 1 and 2) revealing 50 open reading frames which are summarized in Tables I and II. These results represent the first cloning of a gene cluster for enediyne anti-tumor antibiotic biosynthesis.

Table I. Summary of the C-1027 gene cluster open reading frames. Table 1. C-1027 gene cluster open reading frames (-7 to 26), primers for ORF amplification, and proposed functions

orf #	Size	Relative position	Primers	Function	Seq ID No.
orf-(-7)	648 bp	658-11	Fwd: ATG GGC ATG ACG GGT Rev: CTA GAG GAT CCC GGG	very weak homology to putative hydroxylase	3 4
orf-(-6)	549 bp	1478-930	Fwd: ATG CCG CGG ATT CCC Rev: TCA GCT GTC GAT GTC	Viral infectivity potentiator protein	5 6
orf-(-5)	1065 bp	2713-1649	Fwd: ATG ACC ATC GCC ACT Rev: TCA GAG GCC GAG CAC	N-truncated Methionine synthase (likely psuedogene)	7 8
orf-(-4)	387 bp	3238-2851	Fwd: ATG AGC TCG CTA CTG Rev: CTA GGA GCC GGT CGC	Viral transcription factor	9 10
orf-(-3)	1530 bp	4971-3442	Fwd: ATG AGC AGC AGC GCC Rev: TCA TTC GTC GGC TGC	Viral Homolog possibly primase	11 12
orf-(-2)	3027 bp	5982-7478	Fwd: GTG AGG GCT CTG CCG Rev: TCA GAC GGC GGA GGG	Glycerol-Phosphate ABC Transporter (SnoX drug resistance)	13 14
orf-(-1)	2328 bp	9900-7573	Fwd: GTG AGC GTC ACC GAC Rev: TCA ACC CGC CCT GCG	UvrA-like drug resistance pump	15 16
orf-0	1368 bp	11349-9982	Fwd: ATG AGG ATG CTG GTG Rev: GTG GCT GTG CTC GCA	Na^+/H^+ efflux pump	17 18
orf-1	999 bp	28590-29588	Fwd: ATG AGG ATG CTG GTG Rev: TCA GCC GAC GGC GTC	dNTP-glucose dehydratase	19 20
orf-2	1566 bp	29632-31197	Fwd: GTG ACA GCA GTC AAG Rev: TCA TGT GGC CGG TTG	Transmembrane efflux protein	21 22
orf-3	1311 bp	31280-32590	Fwd: GTG GAG TAC TGG AAC Rev: TCA GGC CTG AGG GGC	Coenzyme F390 synthase phenylacetyl-CoA ligase	23 24
orf-4	1584 bp	32809-34392	Fwd: GTG CCC CAC GGT GCA Rev: CTA CAG CCC TCC GAG	phenol hydroxylase chlorophenol-4-monooxygenase	25 26
orf-5	bp	35274-34458	Fwd: ATG TCT TCA ACC CGT Rev: TCA GCC GCG CAG GAA	citrate transport protein	27 28
orf-6	1272	17924-16653	Fwd: ATG CTG GAG AAA TGC Rev: TCA GAC GAG CTC CTT	C-methyl transferase hydroxylase	29 30
orf-	735	16653-	Fwd: ATG GAG TAC GGC CCC	N-	31

7	bp	15919	Rev: TCA TGC CGT GCG CAC	methyltransferase	32
orf-8	1233 bp	15922-14690	Fwd: ATG AGC GGC GGC CCG Rev: TCA CCT CGC CGG ACG	Aminotransferase	33 34
orf-9	432 bp	14643-14212	Fwd: ATG TCG TTA CGT CAC Rev: TCA GCC GAA GGT CAG	CagA	35 36
orf-10	1068 bp	13012-14079	Fwd: ATG AAG GCA CTT GTA Rev: TCA GGC CGC GAT CTC	dNTP-glucose synthase	37 38
orf-11	1485 bp	12835-11351	Fwd: GTG GAC GTG TCA GCG Rev: TCA GGA CCG CGC ACC	Hydroxylase, Halogenase	39 40
orf-12	579 bp	25564-24986	Fwd: ATG AAG CCG ATC GGG Rev: TCAGGA CGA CTT GTT	dNTP-4-keto-6-deoxyglucose 3,5-epimerase	41 42
orf-13	1137 bp	24702-23566	Fwd: ATG CCT TCC CCC TTC Rev: TCA GGT GCG CTC GGC	3-O-acyltransferase	43 44
orf-14	1455 bp	22878-21424	Fwd: GTG AGA GAC GGC CGG Rev:TCA CGT GGT GAT GGC	Coenzyme F-390 Synthase Phenylacetyl CoA Ligase	45 46
orf-15	1482 bp	21407-19926	Fwd: ATG ACC GAC CAG TGC Rev: TCA CAG CAA CTC CTC	Anthranilate Synthase I	47 48
orf-16	663 bp	19929-19267	Fwd: GTG AGC TTG TGG TCT Rev: TCA GGC CGG TTC GGC	Anthranilate Synthase II	49 50
orf-17	1161 bp	19191-18031	Fwd: GTG CGT CCC TTC CGT Rev: TCA GCG GAG CGG ACG	epoxide hydrolase	51 52
orf-18	423 bp	35938-35516	Fwd: ATG CCA GCA CCG ACT Rev: TCA GTC GTT GCC GCG	Unknown	53 54
orf-19	1380 bp	27214-28593	Fwd: ATG CGG GTG ATG ATC Rev: TCA TCG GTC CGC CTC	glycosyl transferase	55 56
orf-20	1356 bp	25815-27170	Fwd: ATG ACC AAG CAC GCC Rev: TCA TAC GGC GGC GCC	squalene monooxygenase	57 58
orf-21	672 bp	23546-22875	Fwd: GTG AGC GCA CAA CTC Rev: TCA CGG CTG TGC CTG	hypothetical Fe-S flavoprotein	59 60
orf-22	816 bp	35274-34458	Fwd: ATG TCT TCA ACC CGT Rev: TCA GCC GCG CAG GAA	haloacetate dehalogenase hydrolase	61 62
orf-23	1380 bp	37559-38938	Fwd: ATG ACG ACG TCC GAC Rev: TCA GGA GGT GAA GGG	peptide synthetase	63 64
orf-24	1620 bp	40986-39367	Fwd: ATG GCA TTG ACT CAA Rev: TCA GCG CAG CTG GAT	Histidine Ammonia lyase	65 66
orf-25	1560 bp	42611-41052	Fwd: ATG ACG CGG CCG GTG Rev: TCA GCG GGT GAG CCG	Type II adenylation protein	67 68
orf-26	282 bp	38983-39264	Fwd: GTG TCC ACC GTT TCC Rev: TCA CTG CGT TCC GGA	Type II peptidyl carrier protein	69 70

Table II. C-1027 gene cluster open reading frames (27 to 42), primers for ORF amplification, and proposed functions

ORF	Relative Position	Primers	Function	SEQ ID NO.
orf-27	43945-46023	Fwd: GTG TGC CCG GTG ACA GAC	Antibiotic	71
		Rev: TCA GCC CAC GGG CTG GGA	Transporter	72
orf-28	46167-47171	Fwd: GTG TTG GGC GAT GAG GAC	O-	73
		Rev: TCA GAC CGC GGA CAT CTG	methyltransferase	74
orf--29	47227-48485	Fwd: ATG GCC GGC CTG GTC ATG	p450	75
		Rev: TCA GGA CCC GAG GGT CAC	hydroxylase	76
orf-30	48610-49714	Fwd: GTG GAC CAG ACG TCT ACG	Oxidoreductase	77
		Rev: TCA TGC AGG TGC AGC GTG		78
orf-31	50350-51390	Fwd: ATG AGG CCG CTC GTT CGG	Unknown	79
		Rev: TCA TCC CGG CCC GGC GGC	Protein	80
orf-32	51420-52341	Fwd: ATG AGA ACG CGG CGA CGC	Oxidoreductase	81
		Rev: TCA CGG CCG GAG GCG TAC		82
orf-33	53241-54074	Fwd: GTG TAT CAG CCG GAC TGT	Unknown	83
		Rev: CTA CTC ATT CCA GTT GTG	Protein	84
orf-34	54230-55379	Fwd: ATG TCT ACG GGC TAT CTC	Unknown	85
		Rev: TCA GCC GCC GGT GGC GCC	Protein	86
orf-35	56027-56881	Fwd: ATG TTC TCC CCC GCC GCC	Oxidase/	87
		Rev: TCA GTA CGC CTG GTG GGC	Dehydrogenase	88
orf-36	56928-57730	Fwd: ATG AAT TCG CTC GAC GAC	Unknown	89
		Rev : TCA GCT CCC GGT CGC CGC	Protein	90
orf-37	57834-58304	Fwd: ATG ACC GCG ACG AAT CCT	Regulatory	91
		Rev: CTA GGC GGC GCG TCC CGC		92
orf-38	58440-60091	Fwd: ATG AGC ACC ACG GCC GAG	Oxidoreductase	93
		Rev: TCA GCC GCG CGC CGA CGG		94
orf-39	60092-60622	Fwd: ATG ACC CTG GAG GCC TAC	Regulatory	95
		Rev: TCA TGC GGG GCT CCC GGT		96
orf-40	60940-62020	Fwd: GTG AAA AGT GAC TCT GCC	Regulatory	97
		Rev: TCA ACG GCG AGT TGG CTG		98
orf-41	62045-62899	Fwd: GTG ACC ACG AAC ACC ATC	Regulatory	99
		Rev: TCA CCC GCG ATC TCG ATC		100
orf-42	62788-63164	Fwd: (partial ORF)	p450	101
		Rev: TCA CCT CGC CGT ACT CAC	hydroxylase	102

Surprisingly, sequence analysis failed to reveal any gene that resembles a polyketide synthase. The C-1027 open reading frames, however, encode polypeptides exhibiting a wide variety of enzymatic activities (*e.g.*, epoxide hydrase, monooxygenase, oxidoreductase, P-450 hydroxylase, *etc.*). The isolated C-1027 gene cluster can be used to

synthesize C-1027 enediyne antibiotics and/or analogues thereof. The C-1027 gene cluster can be modified and/or augmented to increase C-1027 and/or C-1027 analogue production.

Alternatively, various components of the C-1027 gene cluster can be used to synthesize and/or chemically modify a wide variety of metabolites. Thus, for example, ORF 6 (C-methyltransferase) can be used to methylate a carbon, while ORF 12, an epimerase, can be used to change the conformation of a sugar. The ORFs can be combined in their native configuration or in modified configurations to synthesize a wide variety of biomolecules/metabolites. Thus, for example, various combinations of C-1027 open reading frames can be used to synthesize an enediyne core, to synthesize a deoxy sugar, to synthesize a β-amino acid, to make a benzoxazolinate, *etc* (*see, e.g.,* Figures 2, 3, and 4).

The native C-1027 gene cluster ORFs can be re-ordered, modified, and combined with other biosynthetic units (*e.g.* polyketide synthases (PKSs) or catalytic domains thereof and/or non-ribosomal polypeptide synthetases (NRPSs) or catalytic domains thereof) to produce a wide variety of molecules. Large chemical libraries can be produced and then screened for a desired activity.

The C-1027 gene cluster also includes a number of drug resistance genes (*see, e.g.,* Table 2) that confer resistance to C-1027 and/or metabolites involved in C-1027 biosynthesis thereby permitting the cell to complete the enediyne biosynthesis. These resistance genes can be used to confer enediyne resistance on a cell lacking such resistance or to augment the enediyne resistance of a cell that does tolerate enediynes. Such cells can be used to produce high levels of enediynes and/or enediyne metabolites, and/or enediyne analogues.

Table III. C-1027 cluster drug resistance genes.

ORF	Protein	Mechanism
ORF 9:	CagA apoprotein	Drug sequestering
ORF 2:	SgcB transmembrane efflux protein	Drug exporting
ORF 27	Transmembrane transport protein	Drug exporting
ORF 0	Na^+/H^+ transporter	Drug exporting
ORF -1	ABC transport (C-terminus)	Drug exporting
ORF -2	Glycerol phosphate transporter	Drug exporting
ORF -1	UvrA-like protein (N-terminus)	DNA repairing

I. Isolation, preparation, and expression of C-1027 nucleic acids.

The C-1027 gene cluster nucleic acids can be isolated, optionally modified, and inserted into a host cell to create and/or modify a metabolic (biosynthetic) pathway and thereby enable that host cell to synthesize and/or modify various metabolites. Alternatively the C-1027 gene cluster nucleic acids can be expressed in the host cell and the encoded C-1027 polypeptide(s) recovered for use as chemical reagents, *e.g.* in the *ex vivo* synthesis and/or chemical modification of various metabolites. Either application typically entails insertion of one or more nucleic acids encoding one or more isolated and/or modified C-1027 enediyne open reading frames in a suitable host cell. The nucleic acid(s) are typically in an expression vector, a construct containing control elements suitable to direct expression of the C-1027 polypeptides. The expressed C-1027 polypeptides in the host cell then act as components of a metabolic/biosynthetic pathway (in which case the synthetic product of the pathway is typically recovered) or the C-1027 polypeptides themselves are recovered. Using the sequence information provided herein, cloning and expression of C-1027 nucleic acids can be accomplished using routine and well known methods.

A) C-1027 nucleic acids.

The nucleic acids comprising the C-1027 gene cluster are identified in Tables I and are listed in the sequence listing provided herein. In particular, Table 1 identifies genes and functions of open reading frames (ORFs) in the C-1027 enediyne biosynthesis gene cluster and identifies primers suitable for the amplification/isolation of any one or more of the C-1027 open reading frames. Of course, using the sequence information provided herein, other primers suitable for amplification/isolation of one or more C-1027 open reading frames can be determined according to standard methods well known to those of skill in the art (*e.g.* using Vector NTI Suite™, InforMax, Gaithersberg, MD, USA).

Typically such amplifications will utilize the DNA or RNA of an organism containing the requisite genes (*e.g. Streptomyces globisporus*) as a template. Typical amplification conditions include the following PCR temperature program: initial denaturing at 94°C for 5 min, 24-36 cycles of 45 sec at 94°C, 1 min at 60°C, 2 min at 72°C, followed by additional 7 min at 72°C. One of skill will appreciate that optimization of such a protocol, *e.g.* to improve yield, *etc.* is routine (*see, e.g.,* U.S. Patent No. 4,683,202; Innis (1990) *PCR*

Protocols A Guide to Methods and Applications Academic Press Inc. San Diego, CA, *etc*). In addition, primer may be designed to introduce restriction sites and so facilitate cloning of the amplified sequence into a vector.

In one embodiment, this invention provides nucleic acids for the recombinant expression of an enediyne (*e.g.* a C-1027 enediyne or an analogue thereof). Such nucleic acids include isolated gene cluster(s) comprising open reading frames encoding polypeptides sufficient to direct the assembly of the enediyne. In other embodiments of this invention, the C-1027 open reading frames may be unchanged, but the control elements (*e.g.* promoters, enhancers, *etc.*) may be modified. In still other embodiments, the nucleic acids may encode selected components (*e.g.* one or more C-1027 or modified C-1027 open reading frames) and/or may optionally contain other heterologous biosynthetic elements including, but not limited to polyketide synthase (PKS) and/or non-ribosomal polypeptide synthetase (NRPS) modules or enzymatic domains.

Such variations may be introduced by design, for example to modify a known molecule in a specific way, *e.g.* by replacing a single substituent of the enediyne with another, thereby creating a derivative enediyne molecule of predicted structure. Alternatively, variations can be made randomly, for example by making a library of molecular variants of a known enediyne by systematically or haphazardly replacing one or open reading frames in the biosynthetic pathway. Production of alternative/modified enediyne, and hybrid enediyne PKSs and/or NRPSs and hybrid systems is described below.

Using the information provided herein other approaches to cloning the desired sequences will be apparent to those of skill in the art. For example, the enediyne, and/or optionally PKS and/or NRPS modules or enzymatic domains of interest can be obtained from an organism that expresses such, using recombinant methods, such as by screening cDNA or genomic libraries, derived from cells expressing the gene, or by deriving the gene from a vector known to include the same. The gene can then be isolated and combined with other desired biosynthetic elements using standard techniques. If the gene in question is already present in a suitable expression vector, it can be combined *in situ,* with, e.g., other PKS subunits, as desired. The gene of interest can also be produced synthetically, rather than cloned. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in which the sequence will be expressed. The complete sequence can be assembled from overlapping oligonucleotides prepared by standard methods and assembled

into a complete coding sequence (*see, e.g.,* Edge (1981) *Nature* 292:756; Nambair *et al.* (1984) *Science* 223: 1299; Jay *et al.* (1984) *J. Biol. Chem.* 259:6311). In addition, it is noted that custom gene synthesis is commercially available (*see, e.g.* Operon Technologies, Alameda, CA).

Examples of such techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel (1989) *Guide to Molecular Cloning Techniques, Methods in Enzymology 152* Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; Ausubel (19 1994) *Current Protocols in Molecular Biology,* Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., U.S. Patent 5,017,478; and European Patent No. 0,246,864.

B) Expression of f C-1027 open reading frames.

The choice of expression vector depends on the sequence(s) that are to be expressed. Any transducible cloning vector can be used as a cloning vector for the nucleic acid constructs of this invention. However, where large clusters are to be expressed, it phagemids, cosmids, P1s, YACs, BACs, PACs, HACs or similar cloning vectors be used for cloning the nucleotide sequences into the host cell. Phagemids, cosmids, and BACs, for example, are advantageous vectors due to the ability to insert and stably propagate therein larger fragments of DNA than in M13 phage and lambda phage, respectively. Phagemids which will find use in this method generally include hybrids between plasmids and filamentous phage cloning vehicles. Cosmids which will find use in this method generally include lambda phage-based vectors into which cos sites have been inserted. Recipient pool cloning vectors can be any suitable plasmid. The cloning vectors into which pools of mutants are inserted may be identical or may be constructed to harbor and express different genetic markers (see, *e.g.*, Sambrook *et al.*, *supra*). The utility of employing such vectors having different marker genes may be exploited to facilitate a determination of successful transduction.

In preferred embodiments of this invention, vectors are used to introduce C-1027 biosynthesis genes or gene clusters into host (*e.g. Streptomyces*) cells. Numerous vectors for use in particular host cells are well known to those of skill in the art. For example described in Malpartida and Hopwook, (1984) *Nature*, 309:462-464; Kao *et al.*,

(1994), *Science*, 265: 509-512; and Hopwood *et al.*, (1987) *Methods Enzymol.*, 153:116-166 all describe vectors for use in various *Streptomyces* hosts.

In one preferred embodiment, *Streptomyces* vectors are used that include sequences that allow their introduction and maintenance in *E. coli*. Such *Streptomyces/E. coli* shuttle vectors have been described (*see*, for example, Vara *et al.*, (1989) *J. Bacteriol.*, 171:5872-5881; Guilfoile & Hutchinson (1991) *Proc. Natl. Acad. Sci. USA,* 88: 8553-8557.)

The wildtype and/or modified C-1027 enediyne open reading frame(s) of this invention, can be inserted into one or more expression vectors, using methods known to those of skill in the art. Expression vectors will include control sequences operably linked to the desired open reading frame. Suitable expression systems for use with the present invention include systems that function in eucaryotic and/or prokaryotic host cells. However, as explained above, prokaryotic systems are preferred, and in particular, systems compatible with *Streptomyces spp.* are of particular interest. Control elements for use in such systems include promoters, optionally containing operator sequences, and ribosome binding sites. Particularly useful promoters include control sequences derived from enediyne, and/or PKS, and/or NRPS gene clusters. Other promoters (*e.g. ermE** as illustrated in Example 1) are also suitable. Other bacterial promoters, such as those derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose, will also find use in the present constructs. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the beta -lactamase (*bla*) promoter system, bacteriophage lambda PL, and T5. In addition, synthetic promoters, such as the *tac* promoter (U.S. Patent 4,551,433), which do not occur in nature also function in bacterial host cells. In *Streptomyces*, numerous promoters have been described including constitutive promoters, such as *ErmE* and *TcmG* (Shen and Hutchinson, (1994) *J. Biol. Chem.* 269: 30726-30733), as well as controllable promoters such as *actI* and *actIII* (Pleper *et al.*, (1995) *Nature*, vol. 378: 263-266; Pieper *et al.*, (1995) *J. Am. Chem. Soc.*, 117: 11373-11374; and Wiesmann *et al.*, (1995) *Chem. & Biol.* 2: 583-589).

Other regulatory sequences may also be desirable which allow for regulation of expression of the enediyne open reading frame(s) relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

Selectable markers can also be included in the recombinant expression vectors. A variety of markers are known which are useful in selecting for transformed cell lines and generally comprise a gene whose expression confers a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium. Such markers include, for example, genes that confer antibiotic resistance or sensitivity to the plasmid.

The various enediyne cluster open reading frames, and/or PKS, and/or NRPS clusters or subunits of interest can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, *e.g.*, a single promoter. The various open reading frames can include flanking restriction sites to allow for the easy deletion and insertion of other open reading frames so that hybrid synthetic pathways can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

Methods of cloning and expressing large nucleic acids such as gene clusters, including PKS- or NRPS-encoding gene clusters, in cells including *Streptomyces* are well known to those of skill in the art (*see, e.g.,* Stutzman-Engwall and Hutchinson (1989) *Proc. Natl. Acad. Sci. USA,* 86: 3135-3139; Motamedi and Hutchinson (1987) *Proc. Natl. Acad. Sci. USA,* 84: 4445-4449; Grim *et al.* (1994) *Gene,* 151: 1-10; Kao *et al.* (1994) *Science,* 265: 509-512; and Hopwood *et al.* (1987) *Meth. Enzymol.,* 153: 116-166). In some examples, nucleic acid sequences of well over 100kb have been introduced into cells, including prokaryotic cells, using vector-based methods (*see*, for example, Osoegawa *et al.,* (1998) *Genomics*, 52: 1-8; Woon *et al.,* (1998) *Genomics*, 50: 306-316; Huang *et al.,* (1996) *Nucl. Acids Res.*, 24: 4202-4209). In addition, the cloning and expression of C-1027 enediyne is illustrated in Example 1.

C) Host cells.

The vectors described above can be used to express various protein components of the enediyne, and/or enediyne shunt metabolites, and/or other modified metabolites for subsequent isolation and/or to provide a biological synthesis of one or more desired biomolecules (*e.g.* C-1027 and/or a C-1027 analogue, *etc.*). Where one or more proteins of the enediyne biosynthetic gene cluster are expressed (*e.g.* overexpressed) for subsequent isolation and/or characterization, the proteins are expressed in any prokaryotic or

eukaryotic cell suitable for protein expression. In one preferred embodiment, the proteins are expressed in *E. coli.*

Host cells for the recombinant production of the subject enediynes, enediyne metabolites, shunt metabolites, *etc.* can be derived from any organism with the capability of harboring a recombinant enediyne gene cluster and/or subset thereof. Thus, the host cells of the present invention can be derived from either prokaryotic or eucaryotic organisms. Preferred host cells are those of species or strains (*e.g.* bacterial strains) that naturally express enediynes. Such host cells include, but are not limited to *Actinomycetes, Actinoplanetes,* and *Streptomycetes*, *Actinomadura*, *Micromonospra*, and the like. Particularly preferred host cells include, but are not limited to *Streptomyces globisporus*, *Streptomyces lividans*, *Streptomyces coelicolor*, *Micromonospora echinospora* spp. *calichenisis, Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus, and Actinomycete* L585-6. Other suitable host cells include, but are not limited to *S. verticillis S. ambofaciens, S. avermitilis, S. azureus, S. cinnamonensis, S. coelicolor, S. curacoi, S. erythraeus, S. fradiae, S. galilaeus, S. glaucescens, S. hygroscopicus, S. lividans, S. parvulus, S. peucetius, S. rimosus, S. roseofulvus, S. thermotolerans,* and *S. violaceoruber* (*see, e.g.,* Hopwood and Sherman (1990) *Ann. Rev. Genet.* 24: 37-66; *O'Hagan* (1991) *The Polyketide Metabolites,* Ellis Horwood Limited, *etc.*).

In certain embodiments, a eukaryotic host cell is preferred (*e.g.* where certain glycosylation patterns are desired). Suitable eukaryotic host cells are well known to those of skill in the art. Such eukaryotic cells include, but are not limited to yeast cells, insect cells, plant cells, fungal cells, and various mammalian cells (*e.*g. COS, CHO HeLa cells lines and various myeloma cell lines).

D) Recovery of the expression product.

Recovery of the expression product (*e.g.,* enediyne, enediyne analogue, enediyne biosynthetic pathway polypeptide, *etc.*) is accomplished according to standard methods well known to those of skill in the art. Thus, for example where enediyne biosynthetic gene cluster proteins are to be expressed and isolated, the proteins can be expressed with a convenient tag to facilitate isolation (*e.g.* a His_6) tag. Other standard protein purification techniques are suitable and well known to those of skill in the art (*see,*

e.g., (Quadri *et al.* (1998) *Biochemistry* 37: 1585-1595; Nakano *et al.* (1992) *Mol. Gen. Genet.* 232: 313-321, *etc.*).

Similarly where components (*e.g.* enediyne biosynthetic cluster orfs) are used to synthesize and/or modify various biomolecules (*e.g.* enediynes, enediyne analogues, shunt metabolites, *etc.*) the desired product and/or shunt metabolite(s) are isolated according to standard methods well know to those of skill in the art (*see, e.g.*, Carreras and Khosla (1998) *Biochemistry* 37: 2084-2088, Deutscher (1990) *Methods in Enzymology Volume 182: Guide to Protein Purification*, M. Deutscher, ed. *etc.*).

II. Use of C-1027 open reading frames in directed biosynthesis.

Elements (*e.g.* open reading frames) of the C-1027 biosynthetic gene cluster and/or variants thereof can be used in a wide variety of "directed" biosynthetic processes (*i.e.* where the process is designed to modify and/or synthesize one or more particular preselected metabolite(s)). Essentially the entire C-1027 gene cluster can be used to synthesize a C-1027 enediyne and/or a C-1027 enediyne analogue. Individual C-1027 cluster open reading frames can be used to perform chemically modifications on particular substrates and/or to synthesize various metabolites. Thus, for example, ORF 6 (C-methyltransferase can be used to methylate a carbon), while ORF 7 (N-methyltransferase) can be used to methylate a nitrogen. ORF 12, and epimerase, can be used to change the conformation of a sugar, and ORF 8 (an amino transferase) can be used to aminate a suitable substrate. Similarly, combinations of C-1027 open reading frames can be used to direct the synthesis of various metabolites (*e.g.* β-amino acids, deoxysugars, benzoxazolinates, and the like). These examples, are merely illustrative. One of skill in the art, utilizing the information provided here, can perform literally countless chemical modifications and/or syntheses using either "native" enediyne biosynthesis metabolites as the substrate molecule, or other molecules capable of acting as substrates for the particular enzymes in question. Other substrates can be identified by routine screening. Methods of screening enzymes for specific activity against particular substrates are well known to those of skill in the art.

The biosyntheses can be performed *in vivo*, *e.g.* by providing a host cell comprising the desired C-1027 gene cluster open reading frames and/or *in vivo*, *e.g.*, by providing the polypeptides encoded by the C-1027 gene cluster ORFs and the appropriate substrates and/or cofactors.

A) Synthesis of enediynes and enediyne analogues.

In one embodiment, this invention provides for the synthesis of C-1027 enediynes and/or C-1027 analogues or derivatives. In a preferred embodiment, this is accomplished by providing a cell comprising a C-1027 gene cluster and culturing the cell under conditions whereby the desired enediyne or enediyne analogue is synthesized. The cell can be a cell that does not normally synthesize an enediyne and the entire gene cluster can be transfected into the cell. Alternatively, a cell that typically synthesizes enediynes can be utilized and all or part of the C-1027 gene cluster can be introduced into the cell.

Enediyne derivatives/analogues can be produced by varying the order of, or kind of, gene cluster subunits present in the cell, and/or by changing the host cell (*e.g.* to a eukaryotic cell that glycosylates the biosynthetic product), and/or by providing altered metabolites (*e.g.* adding exogenous aglycones to a host that carries a gene cassette of the deoxysugar biosynthesis and glycosylation genes for the production of glycosylated metabolites), *etc.*

In certain embodiments, the host cell need not be transfected with an entire C-1027 gene cluster. Rather, various components of a C-1027 gene cluster can be altered within a cell already harboring a C-1027 cluster. By varying or adding various biosynthetic open reading frames, C-1027 enediyne variants can be produced.

The use of standard techniques of molecular biology (gene disruption, gene replacement, gene supplement) can be used to modulate and/or otherwise alter enediyne and/or other metabolite (*e.g.* shunt metabolite) production in an organism that naturally synthesizes an enediyne (*e.g.* S. globisporus) or an organism that is modified to synthesize an enediyne.

In addition, or alternatively, control sequences that alter the expression of various open reading frames can be introduced that alter the amount and/or timing of enediyne production. Thus, for example, by placing particular C-1027 open reading frames under control of a constitutive promoter (*ermE**) C-1027 production was increased by as much as 4-fold (*see, e.g.* Table 3 and Example 1).

Table 3. Alteration of C-1027 production by engineering the C-1027 biosynthesis gene cluster.

Strain	Yield (%)

WT	100
WT/pKC1139	100
WT/*ermE**/ORF 2	>150
WT/ORF 9	>100
WT/*ermE**/ORF 9	<10
WT/ORF 10, 11	>100
WT/*ermE**/ORF 10, 11	>100
WT/ ORF 9, 10, 11	>400

ORF 2: transmembrane eflux protein; ORF 9: CagA apoprotein; ORF 10: TDP-glucose synthase; ORF 11; Hydroxylase/halogenase

Where enediyne analogues are synthesized, it will often prove desirable to assay them for biological activity. Such assays are well know to those of skill in the art. One such assay is illustrated in Example 1. Briefly, this example depicts an assay of antibacterial activity against *M. luteus* as described by Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579). Other suitable assays for enediyne activity will be known to those of skill in the art.

B) Use of C-1027 open reading frames to synthesize an enediyne core.

The C-1027 open reading frames described herein, or variants thereof, can be used to synthesize an enediyene core, *e.g.,* from a fatty acid precursor. One such synthetic pathway is illustrated in Figure 4. This reaction scheme utilizes ORF 17 (epoxide hydrase), ORF 20 (monooxygenase), ORF 21 (iron-sulfur flavoprotein), ORF 29 (P-450 hydroxylase, ORF 30 (oxidoreductase), ORF 32 (oxidoreductase), ORF 35 (proline oxidase), and ORF 38 (P-450 hydroxylase) to synthesize anenediyne core.

This synthetic pathway, is not considered limiting, but merely illustrative. Using this as a model, one of ordinary skill in the art can design numerous other synthetic schemes to produce enediyne cores and/or core variants.

C) Use of C-1027 open reading frames to synthesize deoxy sugars.

The biosynthesis of various deoxy sugars (*e.g.,* deoxyhexoses) typically share a common key intermediate --4-keto-6-deoxyglucose nucleoside diphosphate or its analogs, whose formation from glucose nucleoside diphosphate is catalyzed by the NGDH enzyme, an NAD^+-dependent oxidoreductase (Liu and Thorson (1994) *Ann. Rev. Microbiol.* 48: 223-256; Piepersberg (1997) pp. 81-163. In *Biotechnology of antibiotics,* 2nd ed. W. R. Strohl

(ed). Marcel Dekker, New York.). Similarly, the C-1027 gene cluster includes an NAGDH enzyme which can be exploited to synthesize a variety of deoxy sugars.

One illustrative synthetic pathway is shown in Figure 2. This biosynthetic scheme utilizes ORF 10 (dNDP-glucose synthase), ORF 1 (dNDP-glucose dehydratase), ORF 12 (epimerase), ORF 8 (aminotransferase), ORF 6 (C-methyltransferase), ORF 7 (N-methyltransferase) and ORF 19 (glycosyl transferase).

This synthetic pathway, is not considered limiting, but merely illustrative. Using this as a model, one of ordinary skill in the art can design numerous other synthetic schemes to produce various deoxy sugars.

D) Use of C-1027 open reading frames to synthesize β-amino acids.

In still another embodiment, C-1027 biosynthetic polypeptides can be used in the biosynthesis of β-amino acids. One illustrative synthetic pathway is shown in Figure 3A. This biosynthetic scheme utilizes ORF 4 (hydroxylase), ORF 11 (hydroxylase/halogenase), ORF 24 (aminomutase), ORF 23 (type II NRPS condensation enzyme), ORF 25 (type II NRPS adenylation enzyme), and ORF 26 (type II peptidyl carrier protein).

Again, this synthetic pathway, is not considered limiting, but merely illustrative. Using this as a model, one of ordinary skill in the art can design numerous other synthetic schemes to produce other beta amino acids.

E) Use of C-1027 open reading frames to synthesize benzoxazolinates.

The C-1027 open reading frames can also be used to synthesize a benzoxazolinate. One illustrative synthetic pathway is shown in Figure 3B. This biosynthetic scheme utilizes ORF 15 (anthranilate synthase I, ORF 16 (anthranilate synthase II), ORF 4 (phenol hydroxylase/chlorophenol-4-monooxygenase), ORF 11 (Hydroxylase/Halogenase), ORF 28 (O-methyltransferase), ORF 3 (coenzyme F390 synthetase, ORF 14 (coenzyme F390 synthetase), and ORF 13 (O-acyltransferase). Again, this synthetic pathway, is not considered limiting, but merely illustrative. Using this as a model, one of ordinary skill in the art can design numerous other synthetic schemes to produce other beta amino acids.

III. Generation of chemical diversity.

In addition to the directed modification and/or biosynthesis of various metabolites as described above, the C-1027 biosynthetic gene cluster open reading frames can be utilized, by themselves or in combination with other biosynthetic subunits (*e.g.* NRPS and/or PKS modules and/or enzymatic domains of other PKS and/or NRPS systems) to produce a wide variety of compounds including, but not limited to various enediyne or enediyne derivatives, various polyketides, polypeptides, polyketide/polypeptide hybrids, various thiazoles, various sugars, various methylated polypeptides/polyketides, and the like.

As with the directed production of various metabolites described above, such compounds can be produced, *in vivo* or *in vitro,* by catalytic biosynthesis, *e.g.,* using large, enediyne cluster units and/or modular PKSs, NRPSs, and hybrid PKS/NRPS systems. In a preferred embodiment large combinatorial libraries of cells harboring various megasynthetases can be produced by the random or directed modification of particular pathways and then selected for the production of a molecule or molecules of interest. It will be appreciated that, in certain embodiments, such libraries of megasynthetases/modified pathways, can be used to generate large, complex combinatorial libraries of compounds which themselves can be screened for a desired activity.

Such combinatorial libraries can be created by the deliberate modification/variation of selected biosynthetic pathways and/or by random/haphazard modification of such pathways.

A) Directed engineering of novel synthetic pathways.

In numerous embodiments of this invention, novel polyketides, polypeptides, and combinations thereof are created by modifying the entediyne gene cluster ORFs and/or known PKSs, and/or NRPSs so as to introduce variations into metabolites synthesized by the enzymes. Such variations may be introduced by design, for example to modify a known molecule in a specific way, *e.g.* by replacing a single monomeric unit within a polymer with another, thereby creating a derivative molecule of predicted structure. Such variations can also be made by adding one or more modules or enzymatic domains to a known PKS or NRPS or enediyne cluster, or by removing one or more module from a known PKS or NRPS.

Using any of these methods, it is possible to introduce PKS domains, NRPS domains, and entediyne domains into a megasynthetase. Mutations can be made to the

native enediyne, and/or NRPS, and/or PKS subunit sequences and such mutants used in place of the native sequence, so long as the mutants are able to function with other subunits (domains) in the synthetic pathway. Such mutations can be made to the native sequences using conventional techniques such as by preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a NRPS and/or PKS subunit using restriction endonuclease digestion. (*see, e.g.,* Kunkel, (1985) *Proc. Natl. Acad. Sci. USA* 82: 448; Geisselsoder *et al.* (1987) *BioTechniques* 5: 786). Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length) which hybridizes to the native nucleotide sequence (generally cDNA corresponding to the RNA sequence), at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located (Zoller and Smith (1983) *Meth, Enzymol.* 100: 468). Primer extension is effected using DNA polymerase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Selection can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations (*see, e.g.,* Dalbie-McFarland *et al.* (1982) *Proc. Natl. Acad. Sci USA* 79:6409). PCR mutagenesis will also find use for effecting the desired mutations.

B) Random modification of enediyne pathways.

In another embodiment, variations can be made randomly, for example by making a library of molecular variants (*e.g.* of a known enediyne) by randomly mutating one or more elements of the subject gene cluster or by randomly replacing one or more open reading frames in a gene cluster with one or more of alternative open reading frames.

The various open reading frames can be combined into a single multi-modular enzyme, thereby dramatically increasing the number of possible combinations obtained using these methods. These combinations can be made using standard recombinant or nucleic acid amplification methods, for example by shuffling nucleic acid sequences encoding various modules or enzymatic domains to create novel arrangements of the sequences, analogous to DNA shuffling methods described in Crameri *et al.* (1998) *Nature* 391: 288-291, and in U.S. Patents 5,605,793 and in 5,837,458. In addition, novel combinations can be made in vitro, for example by combinatorial synthetic methods. Novel molecules or molecule libraries, can be screened for any specific activity using standard methods.

Random mutagenesis of the nucleotide sequences obtained as described above can be accomplished by several different techniques known in the art, such as by altering sequences within restriction endonuclease sites, inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during in vitro DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants or by damaging plasmid DNA in vitro with chemicals. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine intercalating agents such as proflavine, acriflavine, quinacrine, and the like. Generally, plasmid DNA or DNA fragments are treated with chemicals, transformed into *E. coli* and propagated as a pool or library of mutant plasmids.

Large populations of random enzyme variants can be constructed in vivo using "recombination-enhanced mutagenesis." This method employs two or more pools of, for example, 10^6 mutants each of the wild-type encoding nucleotide sequence that are generated using any convenient mutagenesis technique, described more fully above, and then inserted into cloning vectors.

C) Incorporation and/or modification of non-C-1027 cluster elements.

In either the directed or random approaches, nucleic acids encoding novel combinations of gene cluster ORFs are introduced into a cell. In one embodiment, nucleic acids encoding one or more enediyne synthetic cluster ORFS and/or PKS and/or NRPS domains are introduced into a cell so as to replace one or more domains of an endogenous gene cluster within a cell. Endogenous gene replacement can be accomplished using standard methods, such as homologous recombination. Nucleic acids encoding an entire enediyne, enediyne ORF, PKS, NRPS, or combination thereof can also be introduced into a cell so as to enable the cell to produce the novel enzyme, and, consequently, synthesize the novel polymer. In a preferred embodiment, such nucleic acids are introduced into the cell optionally along with a number of additional genes, together called a 'gene cluster,' that influence the expression of the genes, survival of the expressing cells, etc. In a particularly preferred embodiment, such cells do not have any other enediyne and/or PKS- and/or NRPS-encoding genes or gene clusters, thereby allowing the straightforward isolation of the molecule(s) synthesized by the genes introduced into the cell.

Furthermore, the recombinant vector(s) can include genes from a single enediyne and/or PKS and/or NRPS gene cluster, or may comprise hybrid replacement PKS gene clusters with, *e.g.,* a gene for one cluster replaced by the corresponding gene from another gene cluster. For example, it has been found that ACPs are readily interchangeable among different synthases without an effect on product structure. Furthermore, a given KR can recognize and reduce polyketide chains of different chain lengths. Accordingly, these genes are freely interchangeable in the constructs described herein. Thus, the replacement clusters of the present invention can be derived from any combination of PKS and/or NRPS gene sets that ultimately function to produce an identifiable polyketide.

Examples of hybrid replacement clusters include, but are not limited to, clusters with genes derived from two or more of the act gene cluster, the *whiE* gene cluster, frenolicin (*fren*), granaticin (*gra*), tetracenomycin (*tcm*), 6-methylsalicylic acid (6-msas), oxytetracycline (*otc*), tetracycline (*tet*), erythromycin (*ery*), griseusin (*gris*), nanaomycin, medermycin, daunorubicin, tylosin, carbomycin, spiramycin, avermectin, monensin, nonactin, curamycin, rifamycin and candicidin synthase gene clusters, among others. (For a discussion of various PKSs, *see, e.g.,* Hopwood and Sherman (1990) *Ann. Rev. Genet.* 24: 37-66; O'Hagan (1991) The Polyketide Metabolites, Ellis Horwood Limited.

A number of hybrid gene clusters have been constructed, having components derived from the *act, fren, tcm, gris* and *gra* gene clusters (see, e.g., U.S. Patent 5,712,146). Other hybrid gene clusters, as described above, can easily be produced and screened using the disclosure herein, for the production of identifiable polyketides, polypeptides or polyketide/polypeptide hybrids.

Host cells (*e.g. Streptomyces*) can be transformed with one or more vectors, collectively encoding a functional PKS/NRPS set, or a cocktail comprising a random assortment of enediyne ORFs and/or PKS and/or NRPS genes, modules, active sites, or portions thereof. The vector(s) can include native or hybrid combinations of enediyne ORFs, and/or PKS and/or NRPS subunits or cocktail components, or mutants thereof. As explained above, the gene cluster need not correspond to the complete native gene cluster but need only encode the necessary enediyne ORFs and/or PKS and/or NRPS components to catalyze the production of the desired product(s).

IV. Variation of starter and/or extender units, and/or host cells.

In addition to varying the nucleic acids comprising the subject gene cluster, variations in the products produced by the gene cluster(s) can be obtained by varying the the host cell, the starter units and/or the extender units. Thus, for example different fatty acids can be utilized in the enediyne synthetic pathway resulting in different enediyne variants. Similarly different intermediate metabolites can be provided (*e.g.* endogenously produced by the host cell, or produced by an introduced herterologous construct, and/or supplied from an exogenous source (*e.g.* the culture media)). Similarly, varying the host cell can vary the resulting product(s). For example, a gene cassette carrying the enediyne biosynthesis genes can be introduced into a deoxysugar-synthesizing host for the production of glycosylated enediyne metabolites.

V. Use of C-1027 resistance genes.

The antibiotic C-1027 and metabolites present in C-1027 biosynthesis are highly potent cytotoxins. Accordingly the biosynthesis of C-1027 is facilitated by the presence of one or more antibiotic (*e.g.* enediyne) resistance genes. Without being bound to a particular theory, it is believed that CagA and SgcB function cooperatively to provide resistance. It is believed that the C-1027 chromophore is first sequestered by binding to the preaproprotein CagA (ORF 9) to form a complex, which is then transported out of the cell by the efflux pump SgcB (ORF 2) and processed by removing the leader peptide to yield the chromoprotein. Other genes that appear to mediate resistance in the C-1027 biosynthesis gene cluster include a transmembrane transport protein (ORF 27), a Na^+/H^+ transporter (ORF 0), an ABC transporter (ORF -1, C-terminus), a glycerol phosphate transporter (ORF -2), and a UvrA-like protein (ORF -1, N-terminus) (*see, e.g.,* Table 2).

These ORFs and/or the polypeptides encoded by these ORFs can be utilized alone, or in combination with one or more other C-1027 ORFs to confer resistance to enediyne or enediyne metabolites on a cell. This is useful in a wide variety of contexts. For example, to increase production of enediynes. For example, it is believed that C-1027 resistance could be a limiting factor at the onset of C-1027 production. Provision of an extra copy of the plasmid-born *sgcB*, and overexpression of *sgcB* under the control of the constitutive *ermE** promoter resulted in increase of C-1027 production (see example 1).

In a therapeutic context, it is sometimes desirable to confer resistance on certain vulnerable cells. Thus, for example, where an enediyne is used as a

chemotherapeutic, transfection of vulnerable, but healthy cells (*e.g.* liver cells remote from the tumor site, stem cells, *etc.*) with vector(s) expressing the resistance gene(s) permits administration of the enediyne at a higher dosage with fewer adverse effects to the organism. Such approaches have been taken using the multi-drug resistance gene (MDR1) expressing p-glycoprotein.

In another embodiment vectors are provided containing one or more resistance genes of this invention under control of a constitutive and/or inducible promoter thereby providing a "ready-made" expression system suitable for the expression of an enediyne or enediyne metabolite at high concentration.

It is also noted that the resistance genes are expected to confer resistance to compounds other than enediynes. The resistance genes are expected to confer resistance to essentially any cytotoxic compound that can act as a substrate for the resistance gene(s) of this invention.

VI. Kits.

In still another embodiment, this invention provides kits for practice of the methods described herein. In one preferred embodiment, the kits comprise one or more containers containing nucleic acids encoding one or more of the C-1027 biosynthesis gene cluster open reading frames. Certain kits may comprise vectors encoding the *sgc* gene cluster orfs and/or cells containing such vectors. The kits may optionally include any reagents and/or apparatus to facilitate practice of the methods described herein. Such reagents include, but are not limited to buffers, labels, labeled antibodies, bioreactors, cells, *etc.*

In addition, the kits may include instructional materials containing directions (*i.e.,* protocols) for the practice of the methods of this invention. Preferred instructional materials provide protocols utilizing the kit contents for creating or modifying C-1027 gene cluster and/or for synthesizing or modifying a molecule using one or more *sgc* gene cluster ORFs. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Genes for production of the enediyne antitumor antibiotic C-1027 in *Streptomyces globisporus* are clustered with the *cagA* gene that encodes the C-1027 apoprotein

We have been studying the biosynthesis of C-1027 in *Streptomyces globisporus* C-1027 as a model for the enediyne family of antitumor antibiotics (Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188). C-1027 consists of a non-peptidic chromophore and an apoprotein, CagA [also called C-1027AG (Otani *et al.* (1991) *Agri. Biol. Chem.* 55: 407-417)]. The C-1027 chromophore is extremely unstable in the protein-free state, the structure of which was initially deduced from an inactive but more stable degradation product (Minami *et al.* (1993) *Tetrahedron Lett.* 34: 2633-2636) and subsequently confirmed by spectroscopic analysis of the natural product (Yoshida *et al.* (1993) *Tetrahedron Lett.* 34: 2637-2640) (Fig. 1). While the absolute stereochemistry of the deoxysugar moiety was established by total synthesis (Iida *et al.* (1993) *Tetrahedron Lett.* 34: 4079-4082), the *8S, 9S, 13S* and *17R* configuration of the C-1027 chromophore were based only on computer modeling (Okuno *et al.* (1994) *J. Med. Chem.* 37: 2266-2273). Although no biosynthetic study has been carried out specifically on C-1027, the polyketide origin of the enediyne cores has been implicated by feeding experiments with ^{13}C-labeled acetate for the neocarzinostatin chromophore A (Hensens *et al.* (1989) *J. Am. Chem. Soc.* 111: 3295-3299), dynemicin (Tokiwa *et al.* (1992) *J. Am. Chem. Soc.* 114: 4107-4110), and esperamicin (Lam *et al.* (1993) *J. Am. Chem. Soc.* 115: 12340-12345); and deoxysugar biosynthesis has been well characterized in actinomycetes (Liu and Thorson (1994) *Ann. Rev. Microbiol.* 48: 223-256; Piepersberg (1997) pp. 81-163. In *Biotechnology of antibiotics,* 2nd ed. W. R. Strohl (ed). Marcel Dekker, New York). Given the structural similarity of C-1027 to the other enediyne cores and to deoxysugars found in other secondary metabolites, we decided to clone either a PKS or a deoxysugar biosynthesis gene as the first step of identifying the C-1027 gene cluster from *S. globisporus*.

Furthermore, the CagA apoprotein of C-1027 has been isolated, its amino acid sequence has been determined, and the corresponding *cagA* gene has been cloned and sequenced (Otani *et al.* (1991) *Agri. Biol. Chem.* 55: 407-417; Sakata *et al.* (1992) *Biosci.*

Biotech. Biochem. 56: 1592-1595). Since genes encoding secondary metabolite production in actinomycetes have invariably been found to be clustered in one region of the microbial chromosome (Hopwood (1997) *Chem. Rev.* 97: 2465-2497), we further reasoned that mapping the *cagA* gene with either a putative PKS gene, a deoxysugar biosynthesis gene, or both to the same region of the *S. globisporus* chromosome should be viewed as strong evidence supporting the proposition that the cloned genes constitute the C-1027 biosynthesis gene cluster.

We report here the cloning and sequencing of two genes, *sgcA* (*Streptomyces globisporus* C-1027) and *sgcB*, that encode a dNDP-glucose 4,6-dehydratase (NGDH) and a transmembrane efflux protein, respectively. The *sgcA,B* locus is indeed clustered with the *cagA* gene, leading to the localization of a 75-kb gene cluster from *S. globisporus*. The involvement of the cloned gene cluster in C-1027 biosynthesis was demonstrated by disrupting the *sgcA* gene to generate C-1027-nonproducing mutants and by complementing the *sgcA* mutants in vivo to restore C-1027 production. Our results, together with similar effort in the Thorson laboratory on the calicheamicin gene cluster (Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188), represent the first cloning of a gene cluster for enediyne antitumor antibiotic biosynthesis.

Materials and methods.

Bacterial strains and plasmids.

Escherichia coli DH5α was used as a general host for routine subcloning (Sambrook *et al.* (1989) *Molecular cloning, a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). *E. coli* XL 1-Blue MR (Stratagene, La Jolla, CA) was used as the transduction host for cosmid library construction. *E. coli* S17-1 was used as the donor host for *E. coli-S. globisporus* conjugation (Mazodier *et al.* (1989) *J. Bacteriol.* 171: 3583-3585). *Micrococcus luteus* ATCC9431 was used as the testing organism to assay the antibacterial activity of C-1027 (Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579). The pGEM-3zf, -5zf, and -7zf and pGEM-T vectors were from Promega (Madison, WI). *S. globisporus* strains and other plasmids in this study are listed in Table 3

Table 3. Strains and plasmids.

Strain or	Relevant Characteristics

plasmid	
S. globisporus	
C-1027	Wild-type (Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579)
AF40	Mutant resulted from acriflavine treatment of *S. globisporus* C-1027, C-1027-nonproducing (Mao *et al.* (1997) *Chinese J. Biotechnol.* 13: 195-199)
AF44	Mutant resulted from acriflavine treatment of *S. globisporus* C-1027, C-1027-nonproducing (Mao *et al., supra*)
AF67	Mutant resulted from acriflavine treatment of *S. globisporus* C-1027, C-1027-nonproducing (Mao *et al., supra*)
SB1001	*sgcA*-disrupted mutant resulted from integration of pBS1012 into *S. globisporus* C-1027 Apr^R, C-1027-nonproducing
SB1002	*sgcA*-disrupted mutant resulted from integration of pBS1013 into *S. globisporus* C-1027 Apr^R, C-1027-nonproducing

Plasmids:

pOJ446	*E. coli-Streptomyces* shuttle cosmid, Apr^R (Bierman *et al.* (1992) *Gene,* 116: 43-
pOJ260	*E. coli* vector, non-replicating in *Streptomyces*, Apr^R (Bierman *et al. supra)*
pKC1139	*E. coli-Streptomyces* shuttle vector, rep^{TS}, Apr^R (Bierman *et al. supra)*
pWHM3	*E. coli-Streptomyces* shuttle vector, Th^R (Vara *et al.* (1989) *J. Bacteriol.* 171: 5872-5881)
pWHM79	*ermE** promoter in pGEM-3zf (Shen and Hutchinson (1996) *Proc. Natl. Acad. Sci. USA* 93: 6600-6604)
pBS1001	0.75-kb PCR product amplified from *S. globisporus* with type I PKS primers in pGEM-T
pBS1002	0.55-kb PCR product amplified from *S. globisporus* with NGDH gene primers in pGEM-T
pBS1003	0.73-kb PCR product amplified from pBS1005 with *cagA* primers in pGEM-T
pBS1004	pOJ446 *S. globisporus* genomic library cosmid
pBS1005	pOJ446 *S. globisporus* genomic library cosmid
pBS1006	pOJ446 *S. globisporus* genomic library cosmid
pBS1007	3.0-kb *Bam*HI fragment from pBS1005 in pGEM-3zf, *sgcA, sgcB*
pBS1008	4.0-kb *Bam*HI fragment from pBS1005 in pGEM-3zf, *cagA*
pBS1009	1.0-kb *Kpn*I truncated fragment of *sgcA* from pBS1007 in pGEM-3zf
pBS1010	0.75-kb *Sac*II/*Sph*I internal fragment of *sgcA* from pBS1009 in pGEM-5zf

pBS1011	0.75-kb *Sac*I/*Sph*I internal fragment of *sgcA* from pBS1010 in pGEM-3zf
pBS1012	0.75-kb *Eco*RI/*Hind*III internal fragment of *sgcA* from pBS1010 in pOJ260
pBS1013	0.75-kb *Eco*RI/*Hind*III internal fragment of *sgcA* from pBS1010 in pKC1139
pBS1014	2.0-kb *Eco*RI/*Sph*I fragment from pBS1007 in the *Sma*I/*Sph*I sites of pWHM79, *ermE*, sgcA*
pBS1015	2.5-kb *Eco*RI/*Hind*III fragment from pBS1014 in pWHM3, *ermE*, sgcA*
pBS1016	Self-ligation of the 5.2-kb *Kpn*I fragment from pBS1007
pBS1017	0.45-kb *Eco*RI/*Sac*I fragment from pWHM79 in *Eco*RI/*Sac*I sites of pBS1016, *ermE*, sgcB*
pBS1018	2.5-kb EcoRI/HindIII fragment from pBS1017 in pKC1139, *ermE*, sgcB*

Biochemicals and chemicals.

Ampicillin, apramycin, nalidixic acid, and thiostrepton were from Sigma (St. Louis, MO). Unless specified otherwise, restriction enzymes and other molecular biology reagents were from standard commercial sources.

Media and culture conditions.

E. coli strains carrying plasmids were grown in Luria-Bertani (LB) medium and were selected with appropriate antibiotics. *S. globisporus* strains were grown on ISP-4 (Difco Laboratories, Detroit, MI) or R2YE at 28°C for sporulation and in TSB (Hopwood *et al.* (1985) *Genetic manipulation of Streptomyces: a laboratory manual.* John Innes Foundation, Norwich, UK) supplemented with 5 mM $MgCl_2$ and 0.5% glycine at 28°C, 250 rpm for isolation of genomic DNA. For transformation, *S. globisporus* strains were grown in YEME (Hopwood *et al., supra.*) for preparation of protoplasts and on R2YE for protoplast regeneration. For conjugation, both the *E. coli* S17-1 donors and the *S. globisporus* recipients (upon germination in TSB) were prepared in LB, and donors/recipients were grown on either ISP-4 medium with 0.05% yeast extract and 0.1% tryptone or AS-1 medium (Baltz (1980) *Dev. Ind. Microbiol.* 21: 43-54; Bierman *et al.* (1992) *Gene* 116: 43-69) at 30°C for isolation of exconjugants.

For C-1027 production, *S. globisporus* strains were grown either on R2YE or ISP-4 agar medium at 28°C or in liquid medium by a two-stage fermentation. For liquid culture, the seed inoculum was prepared by inoculating 50 mL medium (consisting of 2% glycerol, 2% dextrin, 1% fish meal, 0.5% peptone, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$, pH 7.0) with an aliquot of spore suspension, incubating at 28°C, 250 rpm for two days. To a

fresh 50 mL of the same medium was then added the seed culture (5%), and incubation continued at 28°C, 250 rpm for three to six days (Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579). The fermentation supernatants were harvested by centrifugation (Eppendorf 5415C, 4°C, 10 min, 14,000 rpm) on day 3, 4 and 5, and assayed for their antibacterial activity against *M. luteus* (Hu *et al.* (1988) *J. Antibiot.*, 41: 1575-1579).

DNA isolation and manipulation.

Plasmid preparation and DNA extraction were carried out by using commercial kits (Qiagen, Santa Clarita, CA). Total *S. globisporus* DNA was isolated according to literature protocols (Hopwood *et al.* (1985) *Genetic manipulation of Streptomyces: a laboratory manual.* John Innes Foundation, Norwich, UK; Rao *et al.* (1987) *Methods Enzymol.* 153: 166-198). Restriction endonuclease digestion and ligation followed standard methods (Sambrook *et al.* (1989) *Molecular cloning, a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN).

DNA sequencing.

Automated DNA sequencing was carried out on an ABI Prism 377 DNA Sequencer using the ABI Prism dye terminator cycle sequencing ready reaction kit and AmpliTaq DNA polymerase FS (Perkin-Elmer/ABI, Foster City, CA). Sequencing service was provided by either the DBS Automated DNA Sequencing Facility, UC Davis, or Davis Sequencing Inc. (Davis, CA). Data were analyzed by ABI Prism Sequencing 2.1.1 software and the Genetics Computer Group program (Madison, WI).

Polymerase chain reaction (PCR).

Primers were synthesized at the Protein Structure Laboratory, UC Davis. PCR was carried out on a Gene Amp PCR System 2400 (Perkin-Elmer/ABI) with *Taq* polymerase and buffer from Promega. A typical PCR mixture consisted of 5 ng of *S. globisporus* genomic or plasmid DNA as template, 25 pmoles of each primers, 25 μM dNTP, 5% DMSO, 2 units of *Taq* polymerase, 1 x buffer, with or without 20% glycerol in a final volume of 50 μL. The PCR temperature program was as follows: initial denaturing at 94°C

for 5 min, 24-36 cycles of 45 sec at 94°C, 1 min at 60°C, 2 min at 72°C, followed by additional 7 min at 72°C.

For type II PKS, the following two pairs of degenerate primers were used—5'-AGC TCC ATC AAG TCS ATG RTC GG-3' (forward, SEQ ID NO:103) / 5'-CC GGT GTT SAC SGC GTA GAA CCA GGC G-3' (reverse, SEQ ID NO:104) and 5'-GAC ACV GCN TGY TCB TCV-3' (forward, SEQ ID NO: 105)/5'-RTG SGC RTT VGT NCC RCT-3' (SEQ ID NO:106) (B, C+G+T; N, A+C+G+T; R, A+G; S, C+G; V, A+C+G; Y, C+T) (reverse) (Seow *et al.* (1997) *J. Bacteriol.,* 179: 7360-7368). No product was amplified under all conditions tested. For type I PKS, the following pair of degenerate primers were used—5'-GCS TCC CGS GAC CTG GGC TTC GAC TC-3' (forward, SEQ ID NO:107) / 5'-AG SGA SGA SGA GCA GGC GGT STC SAC-3' (S, G+C) (reverse, SEQ ID NO:108) (Kakavas *et al.* (1997) *J. Bacteriol.,* 179: 7515-7522). A distinctive product with the predicted size of 0.75 kb was amplified in the presence of 20% glycerol and cloned into pGEM-T according to the protocol provided by the manufacturer (Promega) to yield pBS1001.

For NGDH, the following pair of degenerate primers were used—5'-CS GGS GSS GCS GGS TTC ATC GG-3' (forward, SEQ ID NO:109) / 5'-GG GWR CTG GYR SGG SCC GTA GTT G-3' (R, A+G; S, C+G; W, A+T; Y, C+T) (reverse, SEQ ID NO:110) (Decker, *et al.* (1996) FEMS Lett., 141: 195-201). A distinctive product with the predicted size of 0.55 kb was amplified and cloned into pGEM-T to yield pBS1002.

For *cagA*, the following pair of primers, flanking its coding region, were used—5'-AG GTG GAG GCG CTC ACC GAG-3' (forward, SEQ ID NO:111)/5'-G GGC GTC AGG CCG TAA GAA G-3' (reverse, SEQ ID NO:112) (Sakata *et al.* (1992) *Biosci. Biotechnol. Biochem.,* 56: 159201595). A distinctive product with the predicted size of 0.73 kb was amplified from pBS1005 and cloned into pGEM-T to yield pBS1003.

Genomic library construction and screening.

S. globisporus genomic DNA was partially digested with *Mbo*I to yield a smear around 60 kb, as monitored by electrophoresis on a 0.3% agarose gel. This sample was dephosphorylated upon treatment with shrimp alkaline phosphatase and ligated into the *E. coli-Streptomyces* shuttle vector pOJ446 (Bierman *et al.* (1992) *Gene* 116: 43-69) that was prepared by digestion with *Hpa*I, shrimp alkaline phosphatase treatment, and additional digestion with *Bam*HI. The resulting ligation mixture was packaged with the Gigapack II

XL two-component packaging extract (Stratagene). The package mixture was transduced into *E. coli* XL 1-Blue MR. The transduced cells were spread onto LB plates containing apramycin (100 μg/mL) and incubated at 37°C overnight. The titer of the primary library was approximately 6,000 colony-forming units per μg of DNA. Restriction enzyme analysis of twelve randomly selected cosmids confirmed that the average size of inserts was about 35 to 45 kb (Rao *et al.* (1987) *Meth. Enzymol.,* 153: 166-198).

To screen the genomic library, colonies from five LB plates containing apramycin (100 μg/mL, with approximately 2,000 colonies per plate) were transferred to nylon transfer membranes (Micro Separations, Inc., Westborough, MA) and screened by colony hybridization with the PCR-amplified 0.55-kb NGDH fragment from pBS1002 as a probe. The positive cosmid clones were re-screened by PCR with primers for NGDH and confirmed by Southern hybridization (Sambrook *et al., supra.*). Further restriction enzyme mapping and chromosomal walking of these overlapping cosmids led to the genetic localization of the 75-kb *sgc* gene cluster, as represented by pBS1004, pBS1005, and pBS1006 (Fig. 5A). A 3.0-kb *Bam*HI fragment from pBS1005 that hybridized to the NGDH probe was cloned into the same sites of pGEM-3zf to yield pBS1007. Similarly, a 4.0-kb *Bam*HI fragment from pBS1005 that hybridizes to the PCR-amplified 0.73-kb *cagA* probe from pBS1003 was cloned into the same sites of pGEM-3zf to yield pBS1008 (Fig. 5B).

Generation of *sgcA* mutants by insert-directed homologous recombination in *S. globisporus*.

A 1.0-kb *Kpn*I fragment from pBS1007, containing the C-terminal truncated *sgcA*, was subcloned into pGEM-3zf to yield pBS1009. An internal fragment of *sgcA* was moved sequentially as a 0.75-kb *Sac*II/*Sph*I fragment from pBS1009 into the same sites of pGEM-5zf to yield pBS1010 and as a 0.75-kb *Sac*I/*Sph*I fragment from pBS1010 into the same sites of pGEM-3zf to yield pBS1011. The latter plasmid was digested with *Eco*RI and *Hind*III, and the resulting 0.75-kb *Eco*RI/*Hind*III fragment was cloned into the same sites of pOJ260 and pKC1139 (Bierman *et al.* (1992) Gene, 116: 43-69 to yield pBS1012 and pBS1013, respectively.

Introduction of pBS1012 and pBS1013 into *S. globisporus* was carried out by either polyethyleneglycol (PEG)-mediated protoplast transformation (Hopwood *et al.* (1985) *Genetic manipulation of Streptomyces: a laboratory manual.* John Innes Foundation, Norwich, UK) or *E. coli-S. globisporus* conjugation (Bierman *et al.* (1992) *Gene* 116: 43-69;

Matsushima and Baltz (1996) *Microbiology* 142: 261-267; Matsushima *et al.* (1994) *Gene* 146: 39-45), methods for both of which were developed recently in our laboratory. In brief, for transformation, pBS1012 and pBS1013 were propagated in *E. coli* ET12567 (MacNeil *et al.* (1992) *Gene* 111: 61-68), and the resulting double strand plasmid DNA was denatured by alkaline treatment (Ho and Chater (1997) *J. Bacteriol.* 179: 122-127). The latter DNA (5 μL) and 200 μL of 25% PEG 1000 in P buffer (Hopwood *et al. supra*) were sequentially added to 50 μL of *S. globisporus* protoplasts (10^9) in P buffer. The resulting suspension was mixed immediately and spread on R2YE plates. After incubation at 28°C for 16 to 20 hrs, the plates were overlaid with soft R2YE (0.7% agar) containing apramycin (100 μg/mL, final concentration); incubation continued until colonies appeared (in 5 to 7 days). For conjugation, *E. coli* S17-1(pBS1012) or *E. coli* S17-1 (pBS1013) was grown to an OD_{600} of 0.3 to 0.4. Cells from a 20-mL culture were pelleted by centrifugation, washed in LB, and resuspended in 2 mL of LB as the *E. coli* donors. *S. globisporus* spores (10^3 to 10^9) were washed, resuspended in TSB, and incubated at 50°C for 10 min to activate germination. After additional incubation at 37°C for 2 to 5 hrs, the spores were pelleted and resuspended in LB as the *S. globisporus* recipients. The donors (100 μL) and recipients (100 μL) were mixed and spread equally onto two modified ISP-4 or AS-1 plates supplemented freshly with 10 mM $MgCl_2$ (see Media and culture conditions). The plates were incubated at 28°C for 16 to 22 hrs. After removal of most of the *E. coli* S17-1 donors by washing the surface with sterile water, the plates were overlaid with 3 mL of soft LB (0.7% agar) containing nalidixic acid (50 μg/mL, final concentration) and apramycin (100 μg/mL, final concentration) and incubated at 28°C until exconjugants appeared (in approximately 5 days).

Unlike pBS1012, which is a *Streptomyces* non-replicating plasmid, pBS1013 bears a temperature-sensitive *Streptomyces* replication origin (Bierman *et al.* (1992) *Gene* 116: 43-69; Muth *et al.* (1989) *Mol. Gen. Genet.* 219: 341-348) that is unable to replicate at temperatures above 34°C (Table 3), while the *S. globisporus* wild-type strain grows normally up to 37°C. Thus, spores of *S. globisporus* (pBS1013), from either the transformants or the exconjugants, were spread onto R2YE plates containing apramycin (100 μg/mL). The plates were incubated directly at 37°C, and mutants, resulting from single crossover homologous recombination between pBS1013 and the *S. globisporus* chromosome, were readily obtained in 7 to 10 days. Alternatively, the plates were first incubated at 28°C for 2 days until pinpoint-size colonies became visible and then shifted to 37°C to continue incubation.

Mutants resulting from single crossover homologous recombination grew out of the original pinpoint-size colonies as easily distinguishable sectors in 7 to 10 days.

Construction of the *sgcA* and *sgcB* expression plasmids.

pBS1007 was digested with *Eco*RI, and made blunt-ended by treatment with the Klenow fragment of DNA polymerase I. Upon additional digestion with *Sph*I, the resulting 2.0-kb blunt-ended *Sph*I fragment containing the intact *sgcA* gene was cloned into the *Sma*I/*Sph*I sites of pWHM79 (Shen *et al.* (1996) *Proc. Natl. Acad. Sci., USA,* 93: 6600-6604) to yield pBS1014. The latter was digested with *Eco*RI and *Hind*III, and the resulting 2.5-kb *Eco*RI/*Hind*III fragment was cloned into the same sites of pWHM3 (Vara *et al.* (1989) *J. Bacteriol.* 171: 5872-5881) to yield pBS1015, in which the expression of *sgcA* is under the control of the *ermE** promoter (Bibb *et al.* (1994) *Mol. Microbiol.* 14: 533-545).

Alternatively, pBS1007 was digested with *Kpn*I, removing most of the *sgcA* gene, and the 5.2-kb *Kpn*I fragment was recovered and self-ligated to yield pBS1016. The *ermE** promoter was subcloned from pWHM79 (Shen *et al.* (1996) *Proc. Natl. Acad. Sci., USA,* 93: 6600-6604) as a 0.45-kb *Eco*RI/*Sac*I fragment and cloned into the same sites of pBS1016 to yield pBS1017. The latter was digested with *Eco*RI and *Hind*III, and the resulting 2.5-kb *Eco*RI/*Hind*III fragment was cloned into the same sites of pKC1139 to yield pBS1018, in which the expression of *sgcB* is under the control of the *ermE** promoter.

Determination of C-1027 production.

The production of C-1027 was detected by assaying its antibacterial activity against *M. luteus* (Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579). From liquid culture, fermentation supernant (180 μL) was added to stainless steel cylinders placed on LB plates pre-seeded with overnight *M. luteus* culture (0.01% vol/vol). From solid culture, a small square block (0.5 x 0.5 x 0.5 cm^3) of agar from either R2YE or ISP-4 medium was directly placed on *M. luteus*-seeded LB plates. The plates were incubated at 37°C for 24 hrs, and C-1027 production was estimated by measuring the size of inhibition zones.

Nucleotide sequence accession number.

The nucleotide sequence reported here has been deposited in the GenBank database with the accession number AF201913.

Results.

No polyketide synthase gene was amplified by PCR from *S. globisporus*.

On the assumption that the C-1027 enediyne core is of polyketide origin, the PCR approach was adopted to screen *S. globisporus* for any putative PKS genes, although it is far from certain *a priori* if the biosynthesis of the enediyne core invokes a PKS and, if so, whether the enediyne PKS will exhibit a type I or type II structural organization. PCR methods for cloning either type I or type II PKS genes have been developed, and these methods have proven to be very effective in cloning PKS genes from various polyketide-producing actinomycetes (Kakavas *et al.* (1997) *J. Bacteriol.* 179: 7515-7522; Seow *et al.* (1997) *J. Bacteriol.* 179: 7360-7368). While no distinctive product was amplified under all conditions examined with both pairs of primers designed for type II PKS, a single product with the expected size of 0.75 kb was readily amplified by PCR from *S. globisporus* with primers designed for type I PKS, which was subsequently cloned (pBS1001). Intriguingly, sequence analysis of six randomly selected pBS1001 clones yielded an identical product—indicative of a specific PCR amplification—the deduced amino acid sequence of which, however, showed no homology to known PKSs (data not shown), excluding the possibility of using PKS as a probe to identify the *sgc* biosynthesis gene cluster.

Cloning of a putative NGDH gene by PCR from *S. globisporus*.

The biosynthesis of various deoxyhexoses share a common key intermediate—4-keto-6-deoxyglucose nucleoside diphosphate or its analogs—whose formation from glucose nucleoside diphosphate is catalyzed by the NGDH enzyme, an NAD^+-dependent oxidoreductase (Liu and Thorson (1994) *Ann. Rev. Microbiol.* 48: 223-256; Piepersberg (1997) pp. 81-163. In *Biotechnology of antibiotics,* 2nd ed. W. R. Strohl (ed). Marcel Dekker, New York). The PCR method was adopted to clone the putative NGDH gene from *S. globisporus* with primers designed according to the homologous regions of various NGDH enzymes from actinomycetes (Decker *et al.* (1996) *FEMS Lett.* 141: 195-201), resulting in the amplification of a single product with the expected size of 0.55 kb (pBS1002). Sequence analysis of pBS1002 confirmed its identity as a part of a putative NGDH gene.

To clone the complete NGDH gene, an *S. globisporus* genomic library, constructed in the *E. coli-Streptomyces* shuttle vector pOJ446 (Bierman *et al.* (1992) *Gene*

116: 43-69; Rao *et al.* (1987) *Methods Enzymol.* 153: 166-198), was analyzed by Southern hybridization with the PCR-amplified 0.55-kb fragment from pBS1002 as a probe. Of the 10,000 colonies screened, 36 positive colonies were identified, 9 of which were confirmed by PCR to harbor the DGDH gene. Restriction enzyme mapping showed that all of them contained a single 3.0-kb *Bam*HI fragment hybridizing to the NGDH probe. Additional chromosomal walking from this locus eventually led to the localization of the 75-kb *sgc* gene cluster, covered by 18 overlapping cosmids as represented by pBS1004, pBS1005, and pBS1006 (Fig. 5A). The 3.0-kb *Bam*HI fragment was subcloned (pBS1007) (Fig. 5B), and its nucleotide (nt) sequence was determined.

Analysis of the DNA sequences of the *sgcA* and *sgcB* genes.

Two complete open reading frames (ORFs) (*sgcA* and *sgcB*) were identified within the 3.0-kb *Bam*HI fragment of pBS1007, the 3,035-nt sequence of which is shown in Figure 6. The *sgcA* gene most likely begins with an ATG at nt 101, preceded by a probable ribosome biding site (RBS), GGAGG, and ends with a TGA stop codon at nt 1099. S*gcA* should therefore encode a 332-amino acid protein with a molecular weight of 36,341 and an isoelectric point of 6.01. A Gapped-BLAST search showed that the deduced *sgcA* gene product is highly homologous to various putative and known NGDH enzymes from antibiotic-producing actinomycetes, including Gdh from the erythromycin biosynthesis gene cluster in *Saccharopolyspora erythraea* (64% identity and 70% similarity) (Linton *et al.* (1995) *Gene* 153: 33-40), MtmE from the mithramycin biosynthesis gene cluster in *Streptomyces argillaceus* (64% identity and 68% similarity) (Lombo *et al.* (1997) *J. Bacteriol.* 179: 3354-3357), and TylA2 from the tylosin biosynthesis gene cluster in *Streptomyces fradiae* (62% identity and 68% similarity) (Merson-Davies and Cundliffe (1994) *Mol. Microbiol.* 13: 349-355) (Fig. 7). A conserved sequence of 14 amino acid residues close to the N-termini can be easily identified in these proteins, which has been described as a βαβ fold with an NAD^+-binding motif, GxGxxG, (Fig. 7, boxed), consistent with their biochemical role in deoxyhexose biosynthesis (Liu and Thorson (1994) *Ann. Rev. Microbiol.* 48: 223-256; Piepersberg (1997) pp. 81-163. In *Biotechnology of antibiotics,* 2nd ed. W. R. Strohl (ed). Marcel Dekker, New York). The function of Gdh and MtmE as TDP-glucose 4,6-dehydratases, requiring NAD^+ as a cofactor, has been confirmed by an enzyme assay following expression of the *gdh* (Linton *et al.* (1995) *Gene* 153: 33-40) and *mtmE* gene (Lombo *et al.* (1997) *J. Bacteriol.* 179: 3354-3357) in *E. coli*, respectively, and by

purification of the Gdh protein from *Sacc. erythraea* (Vara *et al.* (1989) *J. Bacteriol.* 171: 5872-5881). From these data, it is reasonable to suggest that *sgcA* encodes the NGDH enzyme required for the biosynthesis of the 4,6-dideoxy-4-dimethylamino-5-methylrhamnose moiety of the C-1027 chromophore.

Transcribed in the same direction as *sgcA*, the *sgcB* gene is located 43 nt downstream of *sgcA*. It should begin with a GTG at nt 1143, preceded by a probable RBS, AGGAG, and end with a TGA at nt 2708 (Fig. 6). Correspondingly, *sgcB* should therefore encode a 521-amino acid protein with a molecular weight of 52,952 and an isoelectric point of 4.64. Database comparison of the deduced *sgcB* product revealed that SgcB is closely related to a family of membrane efflux pumps, such as LfrA from *Mycobacterium smegmatis* (43% identity and 50% similarity, protein accession number AAC43550) (Takiff *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93: 362-366), OrfA from *Streptomyces cinnamomeus* (42% identity and 47% similarity, protein accession number AAB71209) (Sommer *et al.* (1997) *Appl. Environ. Microbiol.* 63: 3553-3560), and RifP from the rifamycin biosynthesis gene cluster in *Amycolatopsis mediterranei* (35% identity and 44% similarity, protein accession number AAC01725) Augus *et al.* (1998) *Chem. Biol.* 5: 69-79). These proteins are membrane-localized transporters involved in the transport of antibiotics (conferring resistance), sugars, and other substances. While direct evidence is lacking for RifP conferring rifamycin resistance in *A. mediterranei* by transporting it out of the cells (August *et al.* (1998) *Chem. Biol.,* 5: 68-79), it has been proven that LfrA employs the transmembrane proton gradient in an antiporter mode to drive the efflux of intracellular antibiotics, resulting in fluoroquinolone resistance in *M. smegmatis* (Takiff *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93: 362-366). On the basis of the high degree of amino acid sequence conservation, an equivalent role could be proposed for SgcB, conferring resistance by exporting C-1027 from *S. globisporus*.

The *cagA* gene is clustered with the *sgcA* and *sgcB* locus.

To determine if *cagA* is clustered with the *sgcA* and *sgcB* locus, PCR primers were designed according to the flanking regions of *cagA* (Sakata *et al.* (1992) *Biosci. Biotech. Biochem.* 56: 1592-1595). A single product with the predicted size of 0.73 kb was indeed amplified from several of the overlapping cosmids (which cover the 75-kb *sgc* cluster), including pBS1004 and pBS1005, the identity of which as *cagA* was confirmed by sequencing. Restriction enzyme mapping and Southern hybridization analysis localized

cagA to a single 4.0-kb *Bam*HI fragment that is approximately 14 kb upstream of the *sgcA,B* locus (Fig. 5B). The 4.0-kb *Bam*HI fragment was subcloned (pBS1008), and its nt sequence was determined, revealing the *cagA* gene along with two additional ORFs (data not shown) (Fig. 5). As reported earlier, *cagA* encodes a 142-amino acid protein that is processed by cleavage of a 32-amino acid lead peptide to yield the mature CagA apoprotein (Sakata *et al.* (1992) *Biosci. Biotech. Biochem.* 56: 1592-1595).

Disruption of the *sgcA* gene in *S. globisporus*.

To examine if the cloned *sgc* cluster encodes C-1027 biosynthesis, *sgcA* was insertionally disrupted by a single crossover homologous recombination event to generate C-1027-nonproducing mutant strains (Fig. 8A). Two plasmids were used—pBS1012 (a pOJ260 derivative) and pBS1013 (a pKC1139 derivative), each of which contain a 0.75-kb internal fragment from *sgcA* (Table 3). After introduction of pBS1012 into *S. globisporus* either by PEG-mediated protoplast transformation or *E. coli-S. globisporus* conjugation, transformants or exconjugants that were resistant to apramycin were isolated in all cases. Since pBS1012 is derived from the *Streptomyces* non-replicating plasmid of pOJ260, these isolates must have resulted from integration of pBS1012 into the *S. globisporus* chromosome by homologous recombination. Plasmid pBS1013 was similarly introduced into *S. globisporus*. However, since pBS1013 is derived from pKC1139 that carries the temperature-sensitive *Streptomyces* replication origin from pSG5 and can replicate normally at 28°C (Muth *et al.* (1989) *Mol. Gen. Genet.* 219: 341-348), these isolates were subjected to incubation at the non-permissive temperature of 37°C to eliminate free plasmids from the host cells. As expected, normal growth stopped except for the recombinants that continue to grow at 37°C, indicative of integration of pBS1013 into *S. globisporus* by homologous recombination. The apramycin-resistant *S. globisporus* SB1001 and *S. globisporus* SB1002 strains were chosen as representatives of mutant strains with disrupted *sgcA* gene resulted from integration of pBS1012 and pBS1013, respectively.

To confirm that targeted *sgcA* disruption has occurred by a single crossover homologous recombination event, Southern analysis of the DNA from the mutant strains was performed as exemplified for *S. globisporus* SB1001 with either pOJ260 or the 0.75-kb *Sac*II/*Kpn*I internal fragment of *sgcA* from pBS1010 as a probe. As shown in Fig. 8B, a distinctive band of the predicted size of 6.3 kb was detected with the pOJ260 vector as a probe in all mutant strains (lanes 2, 3, and 4); this band was absent from the wild-type strain

(lane 1). Complementarily, when using the 0.75-kb *Sac*II/*Kpn*I internal fragment of *sgcA* as a probe (Fig. 8C), the 3.0-kb band in the wild-type strain (lane 1) was split into two fragments with the size of 6.3 kb and 1.0 kb in the mutant strains (lanes 2, 3, and 4), as would be expected for disruption of *sgcA* by a single crossover homologous recombination event.

S. globisporus SB1001 and *S. globisporus* SB1002 are C-1027-nonproducing mutants.

No apparent difference in growth characteristics and morphologies between the wild-type *S. globisporus* and mutant *S. globisporus* SB1001 and *S. globisporus* SB1002 strains was observed. While C-1027 production in the wild-type *S. globisporus* strain could be detected on day 3, peaked on day 5, and continued for a few more days, as judged by assaying the antibacterial activity of the culture supernant against *M. luteus* (Hu *et al.* (1988) *J. Antibiot.* 41: 1575-1579), C-1027 production is completely abolished in the *sgcA* mutant strains *S. globisporus* SB1001 and *S. globisporus* SB1002 (Fig. 9A). The latter phenotype was identical to that of the AF40, AF44, and AF67 mutants, C-1027-nonproducing *S. globisporus* strains that have been characterized previously (Fig. 9A and 9C) (Mao, *et al.* (1997) *Chinese J. Biotechnol.* 13: 195-199).

***In vivo* complementation of *S. globisporus* SB1001.**

The ability of the wild-type *sgcA* gene to complement the disrupted *sgcA* gene was tested in the *S. globisporus* SB1001 strain. The construction of pBS1015, in which the expression of *sgcA* is under the control of the constitutive *ermE** promoter, was described in Materials and Methods. Both the pBS1015 construct and the pWHM3 vector as a control were introduced by transformation into the *S. globisporus* SB1001 mutant strains. Culture supernants from each transformant were bioassayed against *M. luteus* for C-1027 production. pBS1015 restored C-1027 production to *S. globisporus* SB1001 to the wild-type level; no C-1027 production was detected in the control in which pWHM3 was introduced into *S. globisporus* BS1001 (Fig. 9B and 9C). A significant reduction of C-1027 production was observed when *S. globisporus* SB1001(pBS1015) was cultured under identical conditions but without thiostrepton (Fig. 9B vs. 6C), indicative that pBS1015 may be unstable in *S. globisporus* SB1001 in the absence of antibiotic selection pressure.

Expression of *sgcB* in *S. globisporus*.

The effect of *sgcB* on C-1027 production was tested in the wild-type *S. globisporus* strain. The construction of pBS1018, in which the expression of *sgcB* is under the control of the constitutive *ermE** promoter, was described in Materials and Methods. pBS1018 and the pKC1139 vector as a control were each introduced by conjugation into *S. globisporus*. Culture supernatants from each exconjugant were harvested on days 3, 4, and 5, and assayed for C-1027 production by determining the antibacterial activity against *M. luteus*. While no apparent difference for C-1027 production was observed between the *S. globisporus* and *S. globisporus* (pKC1139) strains, a significant increase in C-1027 production (150±25%) was evident in the early stage of *S. globisporus* (pBS1018) fermentation (Fig. 9D, day 3). However, such effect on C-1027 production leveled off as the fermentation proceeded and became insignificant when the culture reached the late stationary phase of fermentation (Fig. 9D, day 4 and 5).

Discussion.

Our inability to clone the putative enediyne PKS gene by PCR, with degenerate primers designed according to the highly conserved amino acid sequences of either type I or type II PKSs, or by DNA hybridization, with homologous type I or type II PKS as probes (data not shown), was unexpected, since feeding experiments by incorporation of [1-^{13}C]- and [1,2-^{13}C]acetate into the enediyne cores of esperamicin (Lam *et al.* (1993) *J. Am. Chem. Soc.* 115: 12340-12345), dynemicin (Tokiwa *et al.* (1992) *J. Am. Chem. Soc.* 114: 4107-4110), and neocarzinostatin (Hensens *et al.* (1989) *J. Am. Chem. Soc.* 111: 3295-3299) supported their polyketide origin. Although the enediyne cores are structurally distinct from either the reduced or aromatic polyketides, the biosynthesis of which is well characterized by type I or type II PKS, respectively, it could be imagined that an enediyne PKS catalyzes the biosynthesis of a polyunsaturated linear heptaketide intermediate that is subsequently cyclized into the enediyne core structure (Hu *et al.* (1994) *Mol. Microbiol.* 14: 163-172; Spaink *et al.* (1991) *Nature* 354: 125-130; Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188). Alternatively, Hensens and co-workers proposed a fatty acid origin for the enediyne core that was also consistent with the isotope labeling results. These authors suggested oleate as a precursor that is shortened by loss of carbons from both ends and is desaturated via the oleate-crepenynate pathway to furnish the enediyne core (Hensens *et al.* (1989) *J. Am. Chem. Soc.* 111: 3295-3299). The latter pathway

resembles polyacetylene biosynthesis in higher plants and fungi and requires an acetylene forming enzyme—a plant gene encoding such an enzyme was identified recently (Lee *et al.* (1998) *Science* 280: 915-918). Our DNA sequence analysis of approximately 60 kb of the *sgc* gene cluster, fails to reveal any gene that resembles PKS.

Although little is known about the resistance mechanism for the enediyne antibiotics in general, the apoproteins of the chromoprotein type of enediynes could be viewed as resistance elements that confer self-resistance to the producing organisms by drug sequestration (Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188). Such a resistance mechanism is in fact well established in antibiotic-producing actinomycetes, for example, BlmA, the bleomycin-binding protein from *Streptomyces verticillus* (Shen *et al.* (1999) *Bioorg. Chem.* 27: 155-171). Given the fact that antibiotic production genes have invariably been found to be clustered in one region of the microbial chromosome, consisting of structural, resistance, and regulatory genes, we adopted a strategy to clone the *sgc* gene cluster by mapping a putative C-1027 structural gene to the previously cloned *cagA* gene, considered as a resistance gene that encodes the C-1027 apoprotein.

We chose NGDH as the putative C-1027 structural gene on the basis of the 4,6-dideoxy-4-dimethylamino-5-methylrhamnose moiety of the C-1027 chromophore. It has been well established that all deoxyhexoses could be derived from the common intermediate of 4-keto-6-deoxyglucose nucleoside diphosphate, the biosynthesis of which from glucose nucleoside diphosphate is catalyzed by an NGDH enzyme. We cloned the NGDH gene from *S. globisporus* by PCR and used it as a probe to screen an *S. globisporus* genomic library, resulting in the isolation of the 75-kb *sgc* gene cluster. DNA sequence analysis of a 3.0-kb *Bam*HI fragment of the *sgc* cluster confirmed the presence of the NGDH protein, encoded by *sgcA*, along with *sgcB* that encodes a transmembrane efflux protein (Fig. 6). The *cagA* gene indeed resides approximately 14 kb upstream of *sgcA* (Fig. 5); DNA sequence analysis of a 4.0-kb *Bam*HI fragment confirmed the identity of *cagA* along with two additional ORFs (data not shown). These results underline once again the effectiveness of cloning natural product biosynthesis gene clusters by exploiting the clustering phenomenon between resistance and structural genes.

The involvement of the cloned gene cluster in C-1027 biosynthesis was demonstrated by disrupting the *sgcA* gene to generate *S. globisporus* mutants, the ability of which to produce C-1027 was completely abolished (Fig. 9A), and by complementing the *sgcA* mutants in vivo upon expression of *sgcA* in trans to restore C-1027 production (Fig. 9B

and 6C). These data unambiguously establish that *sgcA* is essential for C-1027 production, and thus support the conclusion that the cloned gene cluster encodes C-1027 biosynthesis. It should be pointed out that, although the *sgcA* mutants *S. globisporus* SB1001 and *S. globisporus* SB1002 were characterized as C-1027-nonproducing on the basis of the antibacterial assay alone (Fig. 9A), this phenotype was identical to that of the controls of the AF40, AF44, and AF67 mutants (Fig. 9A and 9C). The latter strains were isolated previously upon randomly mutagenizing the wild-type *S. globisporus* strain with acriflavine and confirmed to be C-1027-nonproducing by both the antibacterial bioassay and an antitumor spermatogonial assay (Mao, *et al.* (1997) *Chinese J. Biotechnol.* 13: 195-199), providing strong support to the current study. Gene disruption and complementation in *S. globisporus* were made possible by the recently developed genetic system that allowed us to introduce plasmid DNA into *S. globisporus* via either PEG-mediated protoplast transformation (Hopwood *et al.* (1985) *Genetic manipulation of Streptomyces: a laboratory manual.* John Innes Foundation, Norwich, UK) or *E. coli-S. globisporus* conjugation (Bierman *et al.* (1992) *Gene* 116: 43-69; Matsushima and Baltz (1996) *Microbiology* 142: 261-267; Matsushima *et al.* (1994) *Gene* 146: 39-45) for analyzing the *sgc* biosynthesis gene cluster *in vivo*. Given the difficulties encountered with calicheamicin biosynthesis in *Micromonospora echinospora*, into which all attempts to introduce plasmid DNA have failed (Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188), the latter results underscore the importance of selecting C-1027 as a model system for enediyne biosynthesis so that many of the genetic tools developed in *Streptomyces* species can now be directly applied to the study of enediyne biosynthesis.

Finally, the function of *sgcB* was probed by examining C-1027 production, following expression of the gene in the wild-type *S. globisporus* strain. Database comparison of the deduced amino acid sequence clearly suggested SgcB as a transmembrane efflux protein, conferring resistance by exporting C-1027 out of the cell. Hence, in addition to CagA, SgcB could be viewed as the second resistance element identified for C-1027 biosynthesis. Multiple resistance genes have been identified in numerous antibiotic biosynthesis gene clusters (Hopwood (1997) *Chem. Rev.* 97: 2465-2497). It could be imagined that CagA and SgcB function cooperatively to provide resistance—the C-1027 chromophore is first sequestered by binding to the preaproprotein CagA to form a complex, which is then transported out of the cell by the efflux pump SgcB and processed by removing the leader peptide to yield the chromoprotein, although we do not have any experimental

data to substantiate this speculation. Since it is known that yields for antibiotic production could be profoundly altered by the introduction of extra copies of regulatory, resistance, or structural genes into wild-type organisms (Hutchinson (1994) *Bio/Technology* 12: 375-380), we tested the effect of overexpressing *sgcB* in *S. globisporus* on C-1027 production. While no apparent adverse effect on C-1027 production was observed upon introduction of the pKC1139 vector into *S. globisporus* (data not shown), a significant increase in C-1027 production (150±25%) was observed in the early stage of *S. globisporus* (pBS1017) fermentation (Fig. 9D, day 3), supporting the predicted function for SgcB in C-1027 biosynthesis. We propose that C-1027 resistance could be a limiting factor at the onset of C-1027 production, which is circumvented by the extra copy of the plasmid-born *sgcB*, and overexpression of *sgcB* under the control of the constitutive *ermE** promoter results in increase of C-1027 production. However, as the *S. globisporus* (pBS1017) fermentation proceeds to its stationary phase, C-1027 resistance is no longer a limiting factor for overall C-1027 production, and the effect of extra copy of SgcB on C-1027 production consequently became insignificant (Fig. 9D, day 5).

In conclusion, genetic analysis of enediyne biosynthesis has heretofore met with little success in spite of considerable effort (Thorson *et al.* (1999) *Bioorg. Chem.*, 27: 172-188). The localization of the *sgc* gene cluster and characterization of the *sgcA* and *sgcB* genes have now provided an excellent basis for genetic and biochemical investigations and/or modification of C-1027 biosynthesis, and gene disruption and overexpression in *S. globisporus* clearly demonstrated the potential to construct enediyne-overproducing strains and to produce novel enediynes that may have enhanced potency as novel anticancer drugs using combinatorial biosynthesis and targeted mutagenesis. We envisage that the results from C-1027 biosynthesis should facilitate the cloning and characterization of biosynthesis gene clusters of other enediyne antibiotics in *Streptomyces* as well as in other actinomycetes, and could have a great impact on the overall field of combinatorial biosynthesis.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a nucleic acid selected from the group consisting of

a nucleic acid encoding any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA);

a nucleic acid encoding a polypeptide encoded by any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA); and

a nucleic acid amplified by polymerase chain reaction (PCR) using primer pairs that amplify any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA).

2. The isolated nucleic acid of claim 1, wherein said nucleic comprises a nucleic acid encoding at least two open reading frames (ORFs) selected from the group consisting of ORF-1 through ORF 42, excluding ORF 9 (cagA).

3. The isolated nucleic acid of claim 1, wherein said nucleic comprises a nucleic acid encoding at least three open reading frames (ORFs) selected from the group consisting of ORF-1 through ORF 42, excluding ORF 9 (cagA).

4. An isolated nucleic acid comprising a nucleic acid that specifically hybridizes under stringent conditions to an open reading frame (ORF) of the C-1027 biosynthesis gene cluster, excluding ORF 9 (cagA), and can substitute for the ORF to which it specifically hybridizes to direct the synthesis of an enediyne.

5. The isolated nucleic acid of claim 4, wherein said isolated nucleic acid comprises a nucleic acid that specifically hybridizes under stringent conditions to a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, and ORF 14.

6. The isolated nucleic acid of claim 4, wherein said isolated nucleic acid comprises a nucleic acid that specifically hybridizes under stringent conditions to a nucleic

acid selected from the group consisting of ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42.

7. The isolated nucleic acid of claim 5, wherein said isolated nucleic acid comprises a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, and ORF 14.

8. The isolated nucleic acid of claim 6, wherein said isolated nucleic acid comprises a nucleic acid selected from the group consisting of ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42.

9. The isolated nucleic acid of claim 4, wherein said nucleic acid comprises a nucleic acid that is a single nucleotide polymorphism (SNP) of a nucleic acid selected from the group consisting of ORF -7, ORF -6, ORF -5, ORF -4, ORF -3, ORF -2, ORF -1, ORF 0, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 81, ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6, ORF 7, ORF 8, ORF 10, ORF 11, ORF 12, ORF 13, ORF 14, ORF 15, ORF 16, ORF 17, ORF 18, ORF 19, ORF 20, ORF 21, ORF 22, ORF 23, ORF 24, ORF 25, ORF 26, ORF 27, ORF 28, ORF 29, ORF 30, ORF 31, ORF 32, ORF 33, ORF 34, ORF 35, ORF 36, ORF 37, ORF 38, ORF 39, ORF 40, ORF 41, and ORF 42.

10. An isolated gene cluster comprising open reading frames encoding polypeptides sufficient to direct the assembly of a C-1027 enediyne or a C-1027 enediyne analogue.

11. The gene cluster of claim 10, wherein said gene cluster is present in a bacterium.

12. The gene cluster of claim 11, wherein said gene cluster is present in a bacterium selected from the group consisting of *Actinomycetes*, *Actinoplanetes, Actinomadura, Micromonospora, and Streptomycetes.*

13. The gene cluster of claim 11, wherein said gene cluster is present in a bacterium selected from the group consisting *Streptomyces globisporus*, *Streptomyces lividans*, *Streptomyces coelicolor*, *Micromonospora echinospora* spp. *calichenisis*, *Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus, and Actinomycete* L585-6.

14. The gene cluster of claim 13, wherein one or more open reading frames is operatively linked to a heterologous promoter.

15. An isolated polypeptide comprising a catalytic domain encoded by a nucleic acid of a C-1027 gene cluster wherein said nucleic acid comprises a nucleic acid selected from the group consisting of

a nucleic acid encoding any of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA); and

a nucleic acid amplified by polymerase chain reaction (PCR) using any one of the primer pairs identified in Tables I and II that specifically amplify one or more of (ORFs) -7 through 42, excluding ORF 9 (cagA).

16. The polypeptide of claim 15, wherein said polypeptide is encoded by at least two open reading frames selected from the group consisting of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA).

17. The polypeptide of claim 15, wherein said polypeptide is encoded by at least three open reading frames selected from the group consisting of C-1027 open reading frames (ORFs) -7 through 42, excluding ORF 9 (cagA).

18. An expression vector comprising a nucleic acid of any one of claims 1 through 9.

19. A host cell transformed with an expression vector of claim 18.

20. The host cell of claim 19, wherein said cell is transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the assembly of a C-1027 enediyne or a C-1027 enediyne analogue.

21. The host cell of claim 19, wherein said host cell is a bacterium.

22. The host cell of claim 21, wherein said bacterium is selected from the group consisting of Actinomycetes, *Actinoplanetes, Actinomadura, Micromonospora,* and *Streptomycetes.*

23. The host cell of claim 21, wherein said bacterium is selected from the group consisting of *Streptomyces globisporus*, *Streptomyces lividans*, *Streptomyces coelicolor*, *Micromonospora echinospora* spp. *calichenisis, Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus, and Actinomycete* L585-6.

24. A method of chemically modifying a biological molecule, said method comprising contacting a biological molecule that is a substrate for a polypeptide encoded by a C-1027 biosynthesis gene cluster open reading frame, with a polypeptide encoded by a C-1027 biosynthesis gene cluster open reading frame whereby said polypeptide chemically modifies said biological molecule.

25. The method of claim 24, wherein said polypeptide is an enzyme selected from the group consisting of a hydroxylase, a homocysteine synthase, a dNDP-glucose dehydrogenase, a citrate carrier protein, a C-methyl transferase, an N-methyl transferase, an aminotransferase, a CagA apoprotein, an NDP-glucose synthase, an epimerase, an acyl transferase, a coenzyme F390 synthase, and epoxidase hydrolase, an anthranilate synthase, a glycosyl transferase, a monooxygenase, a type II condensation protein, an aminomutase, a type II adenylation protein, an O-methyl transferase, a P-450 hydroxylase, an oxidoreductase, and a proline oxidase.

26. The method of claim 24, wherein said method comprising contacting said biological molecule with at least two different polypeptides encoded by C-1027 biosynthesis gene cluster open reading frames.

27. The method of claim 24, wherein said method comprising contacting said biological molecule with at least three different polypeptides encoded by C-1027 biosynthesis gene cluster open reading frames.

28. The method of claim 24, wherein said contacting is in a host cell.

29. The method of claim 28, wherein said host cell is a bacterium.

30. The method of claim 24, wherein said contacting *ex vivo*.

31. The method of claim 28, wherein said biological molecule is an endogenous metabolite produced by said host cell.

32. The method of claim 28, wherein said biological molecule is an exogenous supplied metabolite.

33. The method of claim 28, wherein said host cell is a eukaryotic cell.

34. The method of claim 33, wherein said eukaryotic cell is selected from the group consisting of a mammalian cell, a yeast cell, a plant cell, a fungal cell, and an insect cell.

35. The method of claim 28, wherein said host cell synthesizes sugars and glycosylates the biological molecule.

36. The method of claim 35, wherein said host cell synthesizes deoxysugars.

37. The method of claim 24, wherein said method further comprises contacting said biological molecule with a polyketide synthase or a non-ribosomal polypeptide synthetase.

38. The method of claim of claim 24, wherein said contacting is in a bacterial cell.

39. The method of claim of claim 24, wherein said contacting is *ex vivo*.

40. The method of claim 24, wherein said method comprises contacting said biological molecule with at substantially all of the polypeptides encoded by C-1027 biosynthesis gene cluster open reading frames and said method produces an enediyne or enediyne analogue.

41. The method of claim 24, wherein said biological molecule is a fatty acid and said biological molecule is contacted with a C-1027 orf polyeptide selected from the

group consisting of an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase.

42. The method of claim 41, wherein said biological molecule is a fatty acid and said biological molecule is contacted with a plurality of C-1027 orf polypeptides comprising an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase.

43. The method of claim 42, wherein said biological molecule is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38.

44. The method of claim 41, wherein said biological molecule is contacted with polypeptides encoded by ORF 15, ORF 16, ORF 28, ORF3, ORF 14, and ORF 13.

45. The method of claim 44 wherein said biological molecule is also contacted with polypeptides encoded by ORF 4 and ORF 3.

46. The method of claim 24, wherein said method comprises contacting a sugar with one or more C-1027 open reading frame polypeptides selected from the group consisting of a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase.

47. The method of claim 46, wherein said method comprises contacting a dNDP-glucose with a plurality of C-1027 open reading frame polypeptides comprising a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase.

48. The method of claim 24, wherein said method comprises contacting an amino acid with one or one or more C-1027 open reading frame polypeptides selected from the group consisting of a hydroxylase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein.

49. The method of claim 48, wherein said method comprises contacting an amino acid with a plurality of C-1027 open reading frame polypeptides comprising a

hydroxylase, a halogenase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein.

50. The method of claim 48, wherein said amino acid is a tyrosine.

51. A method of synthesizing a chromaprotein type enediyne core, said method comprising contacting a fatty acid with one or more C-1027 orf polypeptides selected from the group consisting of an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase.

52. The method of claim 51, wherein said fatty acid is contacted with a plurality of C-1027 orf polypeptides comprising an epoxide hydrase, a monooxygenase, an iron-sulfer flavoprotein, a p-450 hydroxylase, an oxidoreductase, and a proline oxidase.

53. The method of claim 52, wherein said fatty acid is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38.

54. A method of synthesizing a deoxysugar, said method comprising contacting a sugar with one or more C-1027 open reading frame polypeptides selected from the group consisting of a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase.

55. The method of claim 54, wherein said method comprises contacting a dNDP-glucose with a plurality of C-1027 open reading frame polypeptides comprising a dNDP-glucose synthase, a dNDP glucose dehydratase, an epimerase, an aminotransferase, a C-methyltransferase, an N-methyltransferase, and a glycosyl transferase.

56. The method of claim 55, wherein said dNDP-glucose is contacted with polypeptides encoded by ORF17, ORF20, ORF21, ORF29, ORF30, ORF32, ORF35, and ORF38.

57. A method of synthesizing a beta amino acid, said method comprising contacting an amino acid with one or one or more C-1027 open reading frame polypeptides selected from the group consisting of a hydroxylase, an aminomutase, a type II NRPS

condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein.

58. The method of claim 57, wherein said method comprises contacting an amino acid with a plurality of C-1027 open reading frame polypeptides comprising a hydroxylase, a halogenase, an aminomutase, a type II NRPS condensation enzyme, a type II NRPS adenylation enzyme, and a type II peptidyl carrier protein.

59. The method of claim wherein said amino acid is contacted with polypeptides encoded by ORF 4, ORF11, ORF24, ORF23, ORF25, and ORF26.

60. The method of claim 57, wherein said amino acid is a tyrosine.

61. A method of synthesizing an enediyne or an enediyne analogue said method comprising:

culturing a cell comprising a recombinantly modified C-1027 gene cluster under conditions whereby said cell expresses said enediyne or enediyne analogue; and

recovering said enediyne or enediyne analogue.

62. The method of claim 61, wherein said gene cluster is present in a bacterium.

63. The gene cluster of claim 62, wherein said gene cluster is present in a bacterium selected from the group consisting of Actinomycetes, *Actinoplanetes, Actinomadura, Micromonospora,* and *Streptomycetes.*

64. The gene cluster of claim 62, wherein said gene cluster is present in a bacterium selected from the group consisting *Streptomyces globisporus*, *Streptomyces lividans*, *Streptomyces coelicolor*, *Micromonospora echinospora* spp. *calichenisis, Actinomadura verrucosopora, Micromonospora chersina, Streptomyces carzinostaticus, and Actinomycete* L585-6.

65. The method of claim 61, wherein said gene cluster is present in a eukaryotic cell.

66. The method of claim 65, wherein said eukaryotic cell is selected from the group consisting of a mammalian cell, a yeast cell, a plant cell, a fungal cell, and an insect cell.

67. The method of claim 61, wherein said host cell synthesizes sugars and glycosylates said enediyne or enediyne analogue.

68. The method of claim 67, wherein said host cell synthesizes deoxysugars.

69. A method of making a cell resistant to an enediyne or an enediyne metabolite, said method comprising expressing in said cell one or more isolated C-1027 open reading frame nucleic acids that encode a protein selected from the group consisting of a CagA apoprotein, a SgcB transmembrane efflux protein, a transmembrane transport protein, a Na+/H+ transporter, an ABC transport, a glycerol phosphate tranporter, and a UvrA-like protein.

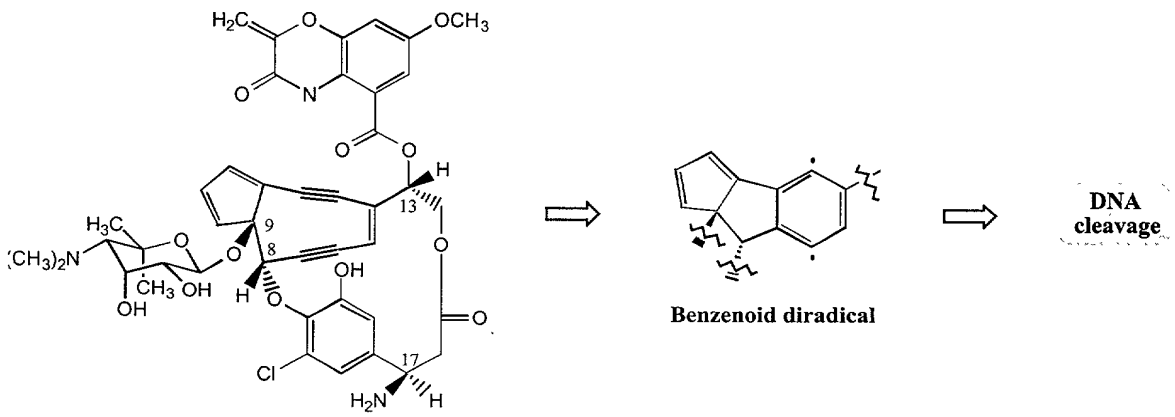
70. The method of claim 69, wherein said isolated C-1027 open reading frame nucleic acids are selected from the group consisting of ORF 9, ORF2, ORF 27, ORF 0, ORF 1 c-terminus, ORF 2, and ORF 1 N-terminus.

71. The method of claim 69, wherein said cell is a bacterial cell.

GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTIC C-1027

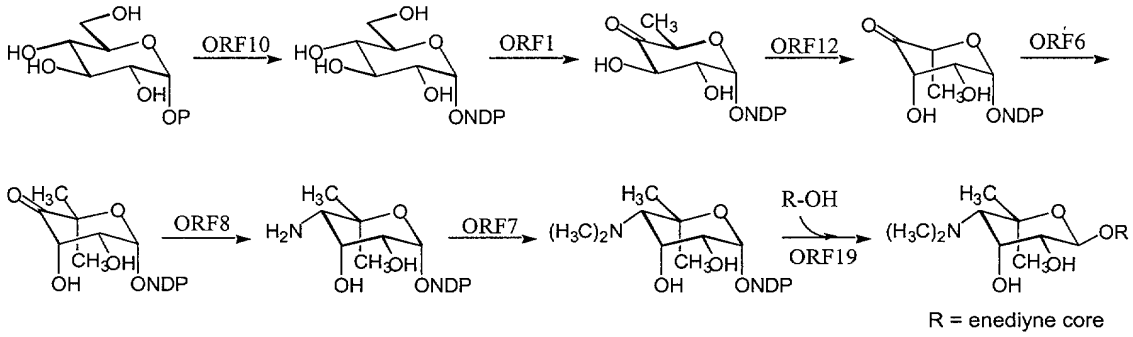
ABSTRACT OF THE DISCLOSURE

This invention provides nucleic acid sequences and characterization of the gene cluster responsible for the biosynthesis of the enediyne C-1027 (produced by *Streptomyces globisporus*). Methods are provided for the biosynthesis of enediynes, enediyne analogs and other biological molecules.



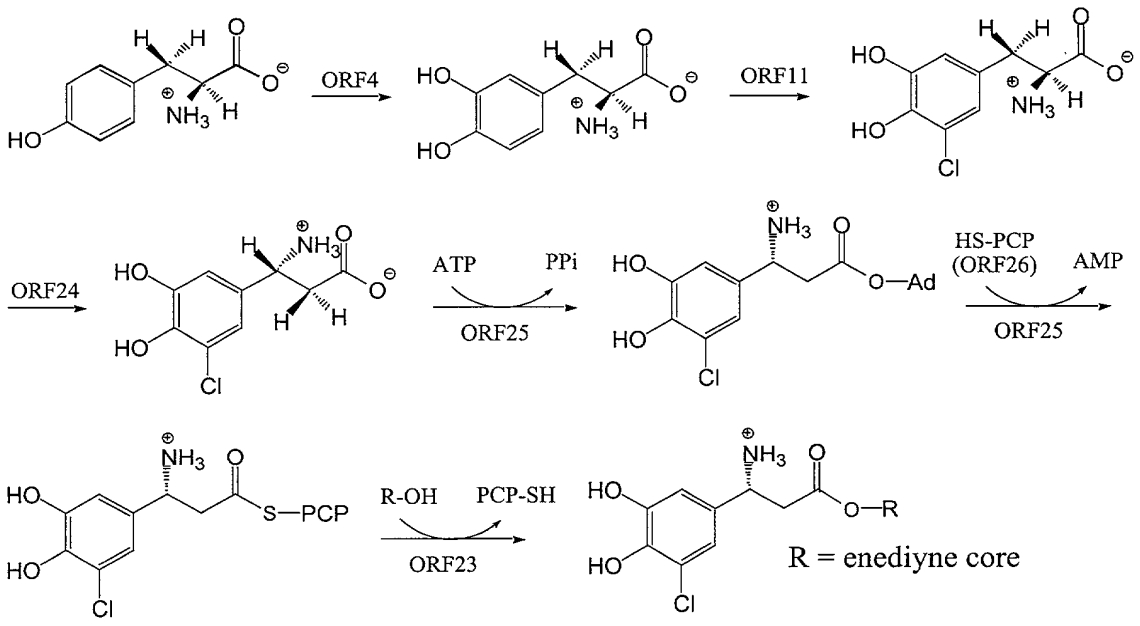
C-1027 chromophore

Fig. 1



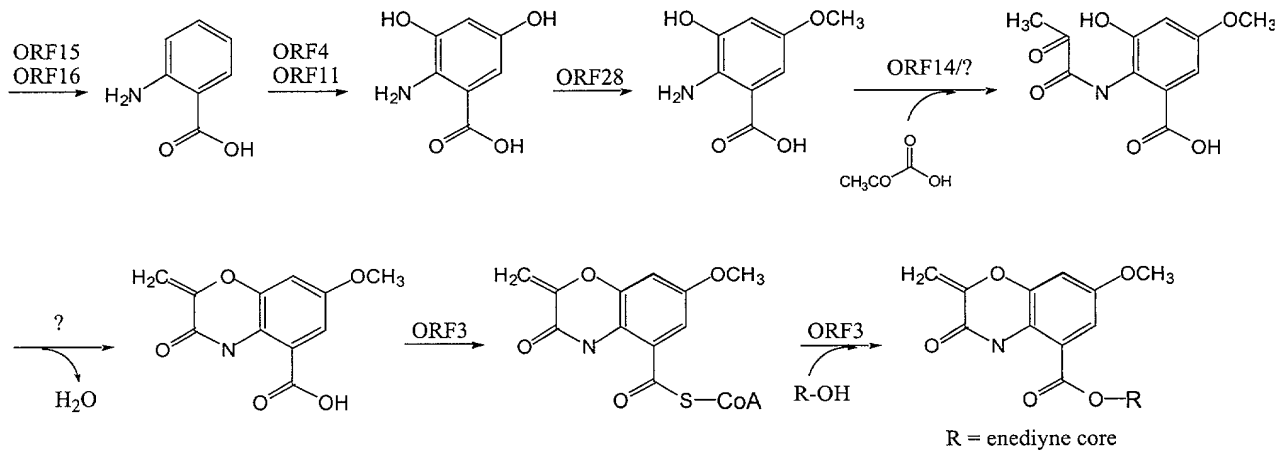
ORF10: dNDP-glucose synthase, 355 aa
ORF1: dNDP-glucose dehydratase, 332 aa
ORF12: epimerase, 192 aa
ORF8: aminotransferase, 410 aa
ORF6: C-methyltransferase, 423 aa
ORF7: N-methyltransferase, 244 aa
ORF19: glycosyl transferase, 459 aa

Fig. 2



ORF4: Hydroxylase, 527 aa
ORF11: Hydroxylase/halogenase, 492/494 aa
ORF24: Aminomutase, 539 aa
ORF23: Type II NRPS condensation enzyme, 459 aa
ORF25: Type II NRPS adenylation enzyme, 716 aa
ORF26: Type II peptidyl carrier protein, 93 aa

Fig. 3A



ORF15: Anthranilate synthase I, 493 aa	**ORF3: Coenzyme F390 synthetase, 463 aa**
ORF16: Anthranilate synthase II, 220 aa	**ORF14: Coenzyme F390 synthetase, 484 aa**
ORF28: O-methyltransferase, 350 aa	**ORF13: O-acyltransferase, 378 aa**

Fig. 3B

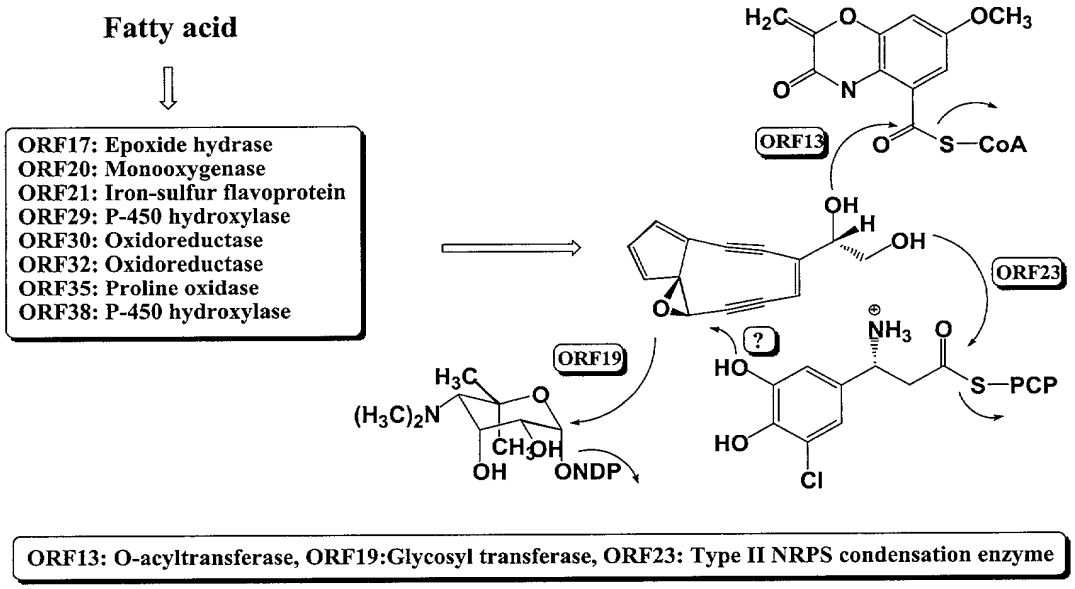

Fatty acid
ORF17: Epoxide hydrase
ORF20: Monooxygenase
ORF21: Iron-sulfur flavoprotein
ORF29: P-450 hydroxylase
ORF30: Oxidoreductase
ORF32: Oxidoreductase
ORF35: Proline oxidase
ORF38: P-450 hydroxylase
ORF13
ORF19
ORF23
?
S—CoA
S—PCP
ONDP
ORF13: O-acyltransferase, ORF19:Glycosyl transferase, ORF23: Type II NRPS condensation enzyme

Fig. 4

Fig. 5A

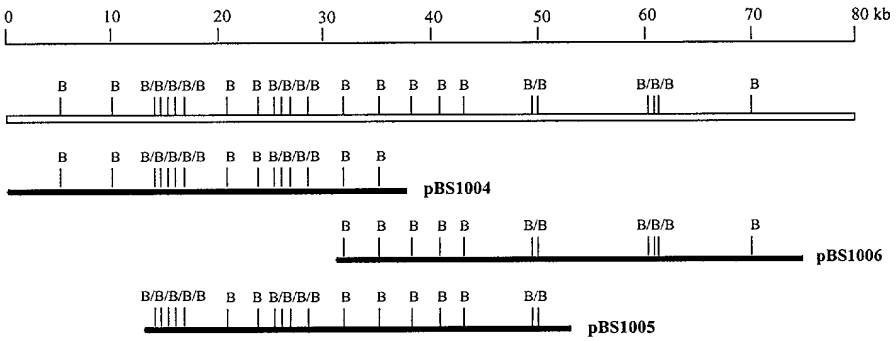


Fig. 5B

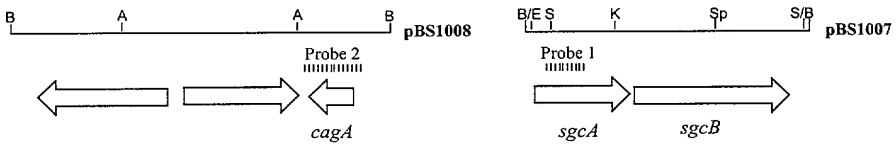


Fig. 5C

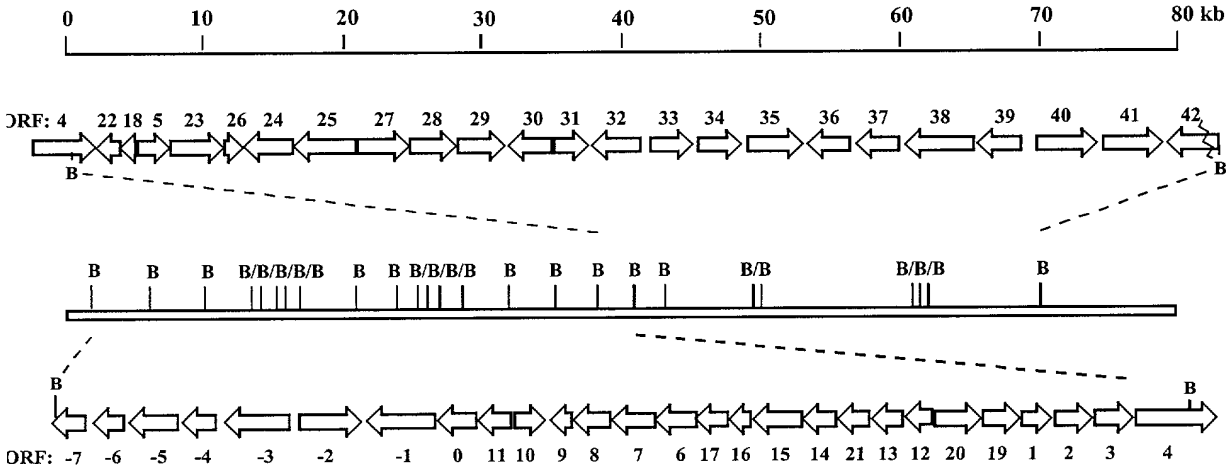


Fig. 6

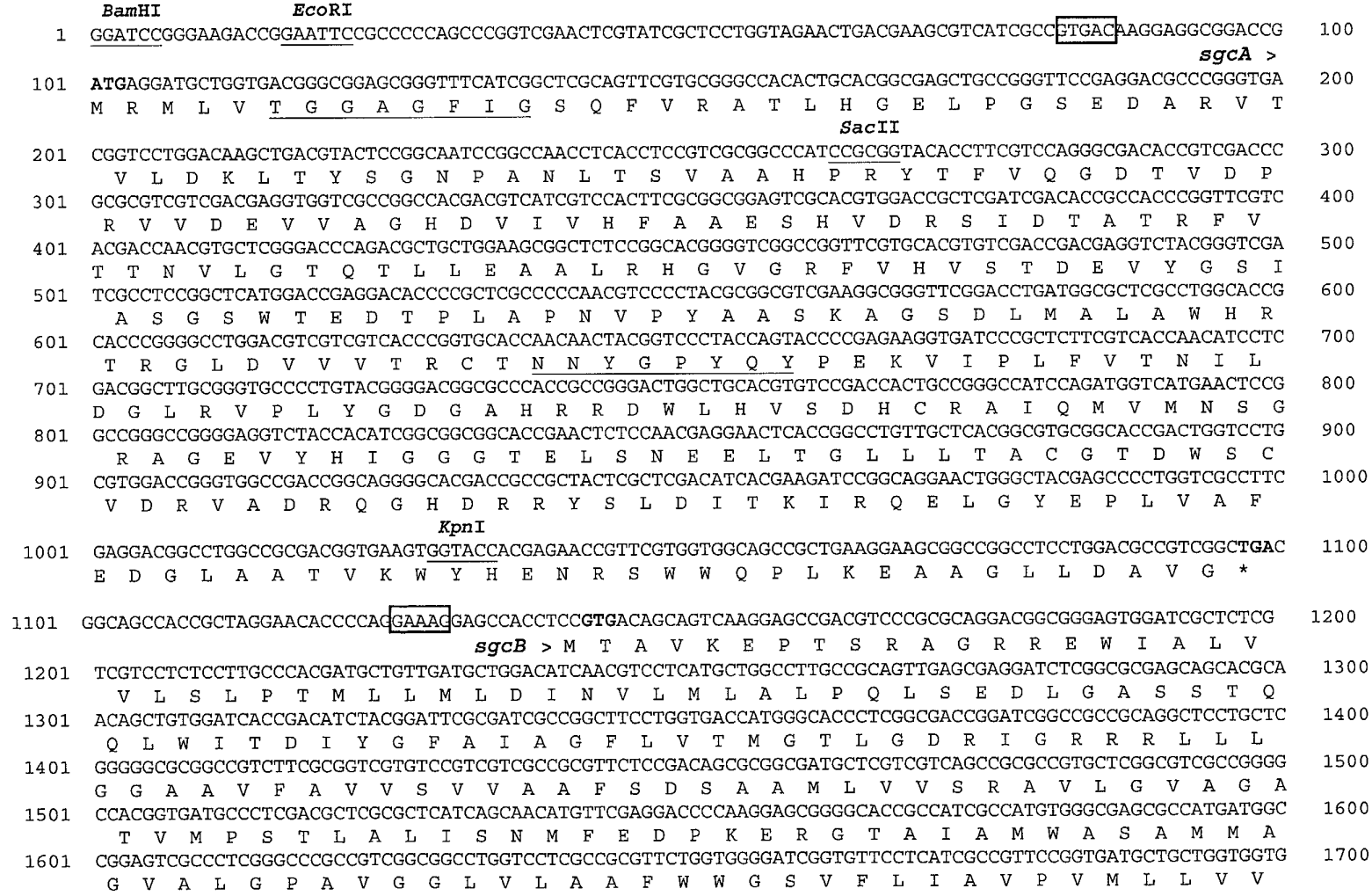


Fig. 6 cont'd.

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1801  TCTACGGACTGAAGGAGCTGGCCCGGACCGGGTGGGACCCGCTCGCCGCCGGCGCGGTGGTCCTCGGCGTGATCTTCGGCGCGCTGTTCGTCCAGCGCCA  1900
        Y  G  L  K  E  L  A  R  T  G  W  D  P  L  A  A  G  A  V  V  L  G  V  I  F  G  A  L  F  V  Q  R  Q
1901  GCGGCGGTTGGCCGACCCCATGCTGGACCTCGGCCTCTTCGCCGACCGCACCCTGCGGGCGGGTCTGACGGTCAGTCTGGTCAACGCCGTCATCATGGGC  2000
       R  R  L  A  D  P  M  L  D  L  G  L  F  A  D  R  T  L  R  A  G  L  T  V  S  L  V  N  A  V  I  M  G
                                                                                    SphI
2001  GGGACCGGACTGATGGTCGCCCTGTACCTCCAGACGATCGCCGGTCACTCCCCGTTGGCCGCCGGGCTGTGGCTGCTGATCCCGGCCTGCATGCTCGTCG  2100
      G  T  G  L  M  V  A  L  Y  L  Q  T  I  A  G  H  S  P  L  A  A  G  L  W  L  L  I  P  A  C  M  L  V  V
2101  TGGGCGTACAGCTGTCGAACCTGCTGGCCCAGCGGATGCCCCCTTCCCGGGTGCTGCTGGGGGGACTGCTGATCGCGGCCGTCGGACAGCTCCTGATCAC  2200
        G  V  Q  L  S  N  L  L  A  Q  R  M  P  P  S  R  V  L  L  G  G  L  L  I  A  A  V  G  Q  L  L  I  T
2201  CCAGGTGGACACCGAGGACACCGCCCTCCTCATCGCGGCCACCACCCTGATCTACTTCGGCGCCTCACCGGTGGGGCCGATCACCACGGGCGCGATCATG  2300
       Q  V  D  T  E  D  T  A  L  L  I  A  A  T  T  L  I  Y  F  G  A  S  P  V  G  P  I  T  T  G  A  I  M
2301  GGAGCCGCGCCCCCGGAGAAGGCGGGTGCCGCCTCGTCGCTGTCCGCCACCGGCGGCGAGTTCGGAGTGGCGCTCGGCATCGCGGGCCTGGGGAGTCTGG  2400
      G  A  A  P  P  E  K  A  G  A  A  S  S  L  S  A  T  G  G  E  F  G  V  A  L  G  I  A  G  L  G  S  L  G
2401  GCACCGTCGTGTACAGCGCCGGGGTCGAGGTGCCGGACGCGGCCGGGCCCGCCGACGCCGACGCCGCGCAGGAGAGCATCGCCGGCGCCCTGCACACGGC  2500
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2501  CGGTCAGCTGGCACCGGGCAGCGCCGACGCCCTGCTGGACTCCGCGCGCGCGGCCTTCACCAGCGGCGTGCAGTCCGTCGCCGCCGTCTGCGCCGTGTTC  2600
       G  Q  L  A  P  G  S  A  D  A  L  L  D  S  A  R  A  A  F  T  S  G  V  Q  S  V  A  A  V  C  A  V  F
2601  TCCCTGGCGCTCGCCGTCCTCATCGGCACCCGGCTGCGGGACATTTCCGCGATGGACCACGGGCACGGCGAGGAACCGGCCGAGAACGACGCTCAACCGG  2700
      S  L  A  L  A  V  L  I  G  T  R  L  R  D  I  S  A  M  D  H  G  H  G  E  E  P  A  E  N  D  A  Q  P  A
2701  CCACATGAGCGCACTTCCGGAGATGCAACGGCCGCCGTCGAGGTATGAGGATCACCTTCCGGGGTGCACCTGCACGGCAACGGAGGCGTAGTGGAGTACT  2800
        T  *
2801  GGAACAGCACGGCGGAGACCATGCCCCGCCAGGAACTCGAACAGTGGAAGTGGCGCAGGCTCCAGGCCGCCATGGACCACGCCAGAAGGCTTTCGCCCTT  2900
                                                                                    SacII
2901  CTGGCGGGAACGACTCCCCGAGAACATCACCTCCATGGCGGACTACGCGGCGCGGGTGCCTCTCCTGCGCAAGGCCGACCTCCTCGCCGCGGAAGCCGCG  3000
                                  BamHI
3001  TCTCCCCCTTACGGCACCTGGCCCTCGCTGGATCC                                                                  3035
```

Fig. 7

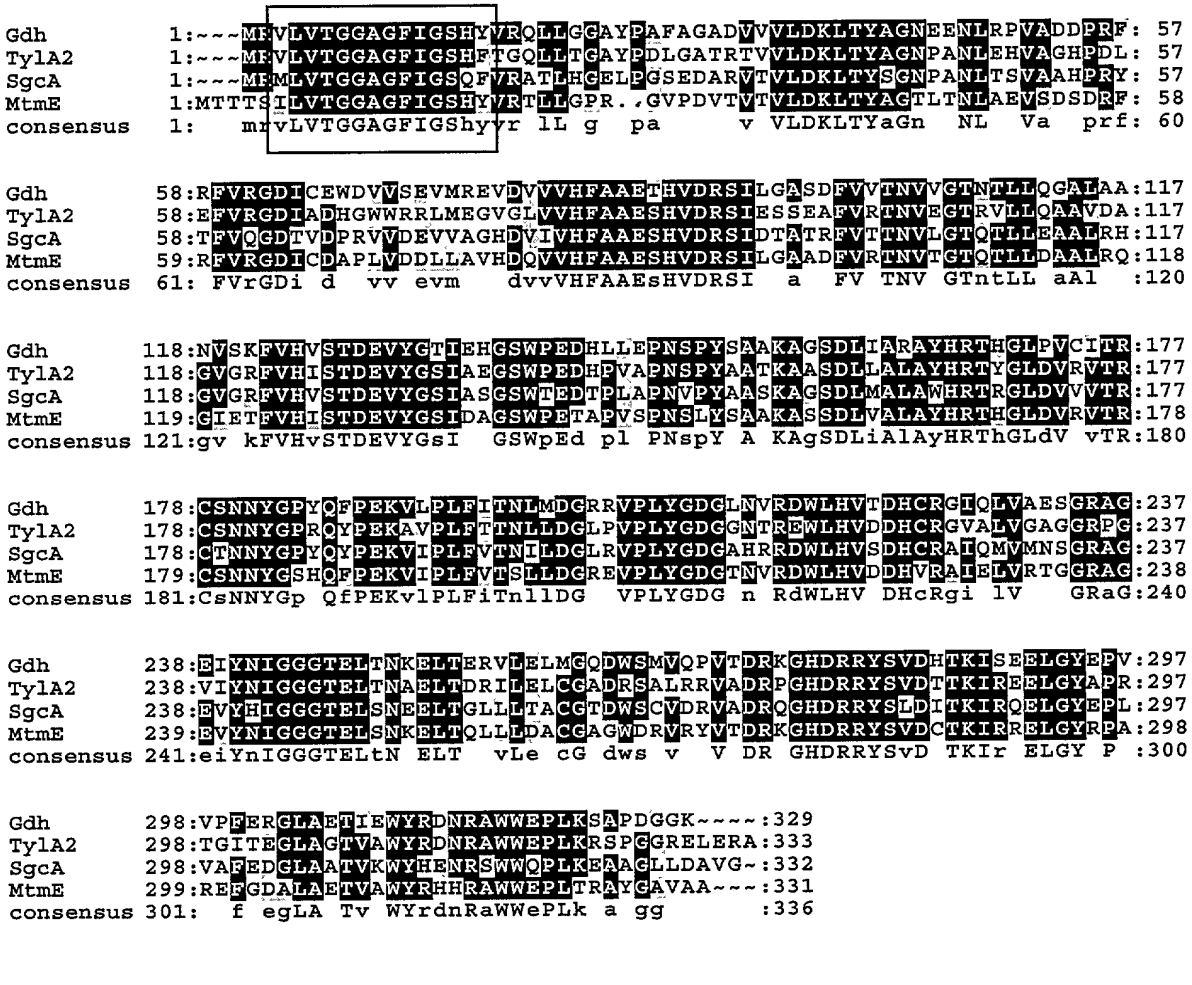
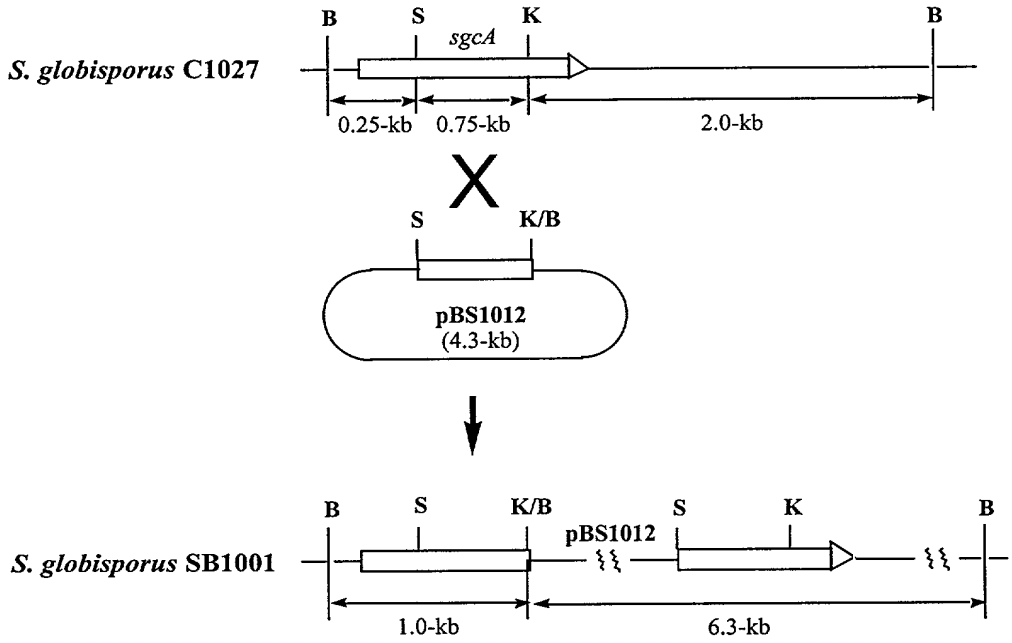


Fig. 8A



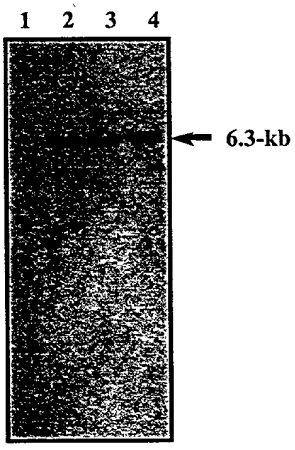


Fig. 8B

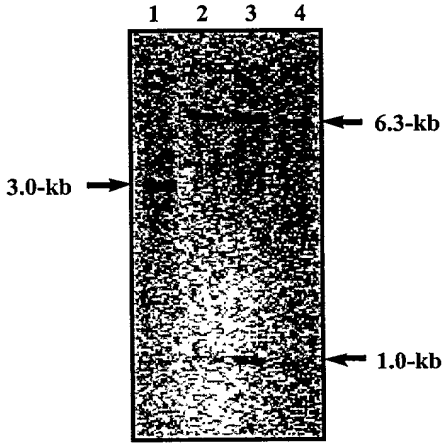


Fig. 8C

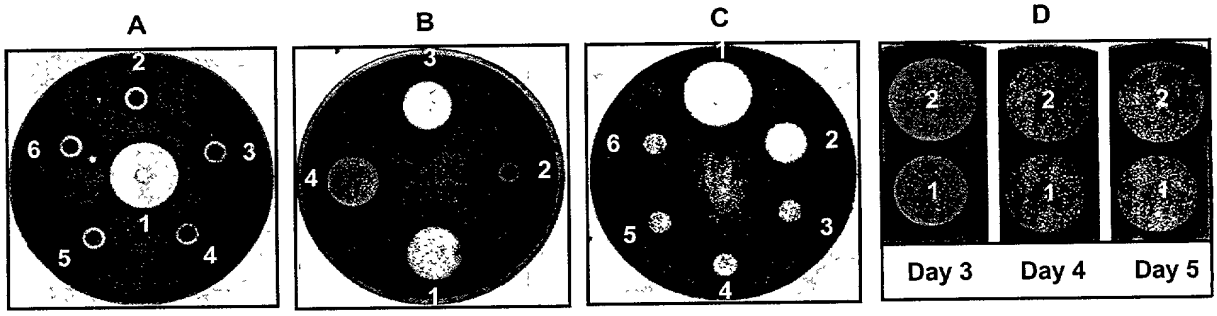


Fig. 9A *Fig. 9B* *Fig. 9C* *Fig. 9D*

PATENT APPLICATION DECLARATION

(Attorney's Docket No.: 2500.125US2)

Each of the Applicants named below hereby declares as follows:

1. My residence, post office address and country of citizenship given below are true and correct.

2. I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought in the patent application entitled "GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTIC C-1027," Serial No. _____, filed January 5, 2000, and I have reviewed and understand the contents of the specification, including its claims.

3. I acknowledge my duty to disclose to the Office all information known to me to be material to patentability of this application, in accordance with 37 C.F.R. Section 1.56, which is defined on the attached page.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____ _____

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Date: _____ _____

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(Citizenship: United States)

Date: _____ _____

Scott Standage
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(Citizenship: United Kingdom)

Section 1.56 Duty to Disclose Information Material to Patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application;

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

GENE CLUSTER FOR PRODUCTION OF THE ENEDIYNE ANTITUMOR ANTIBIOTICC-1027

SEQUENCE LISTING

SEQ ID No. 1. C-1027 gene cluster DNA sequence from 1 to 42,000, ORF-(-7) to ORF-26

```
          GTCGACTCTAGAGGATCCCGGGTGCGGAGTAGGGGTTACGGACGAAGGAGGGGTGCCCGG
        1 ---------+---------+---------+---------+---------+---------+ 60
          CAGCTGAGATCTCCTAGGGCCCACGCCTCATCCCCAATGCCTGCTTCCTCCCCACGGGCC
-7-*                    *  L  I  G  P  A  S  Y  P  N  R  V  F  S  P  H  G   -

          CGACGCCTGCGGCGAAGGGCGGTTCCTTGAGTTCGAGGCCGGTGGCGAGGACGACGTGGT
       61 ---------+---------+---------+---------+---------+---------+ 120
          GCTGCGGACGCCGCTTCCCGCCAAGGAACTCAAGCTCCGGCCACCGCTCCTGCTGCACCA
-7        A  V  G  A  A  F  P  P  E  K  L  E  L  G  T  A  L  V  V  H   -

          CCGCGTCGAGGATCTGCGTGTCGGGGAGCGGCCCAGGGCGCAGCCCCTCGGTCAGGTACG
      121 ---------+---------+---------+---------+---------+---------+ 180
          GGCGCAGCTCCTAGACGCACAGCCCCTCGCCGGGTCCCGCGTCGGGGAGCCAGTCCATGC
-7        D  A  D  L  I  Q  T  D  P  L  P  G  P  R  L  G  E  T  L  Y   -

          GGGTGAGGCCCCTGACGGTCACCTCGAAGCAGCGGTCGTGGGACCGGGCGTCGAGCGCCT
      181 ---------+---------+---------+---------+---------+---------+ 240
          CCCACTCCGGGGACTGCCAGTGGAGCTTCGTCGCCAGCACCCTGGCCCGCAGCTCGCGGA
-7        P  T  L  G  R  V  T  V  E  F  C  R  D  H  S  R  A  D  L  A   -

          CCCCGTCCGCTTCCACAAGGACGACGCCGGGACAGGACTCCCGTGCGGCCTCGACCAGTC
      241 ---------+---------+---------+---------+---------+---------+ 300
          GGGGCAGGCGAAGGTGTTCCTGCTGCGGCCCTGTCCTGAGGGCACGCCGGAGCTGGTCAG
-7        E  G  D  A  E  V  L  V  V  G  P  C  S  E  R  A  A  E  V  L   -

          GGGCGTCGAGGTAGTCCTGGAAGATGCGGCGGGGGGCGGGGCCCTGTTCGGTGAACTTCC
      301 ---------+---------+---------+---------+---------+---------+ 360
          CCCGCAGCTCCATCAGGACCTTCTACGCCGCCCCCCGCCCCGGGACAAGCCACTTGAAGG
-7        R  A  D  L  Y  D  Q  F  I  R  R  P  A  P  G  Q  E  T  F  K   -

          ACGAAGCCCAGCGCCGGGGCCAGTCGCGCCGGTCGGCCTCCTGGTTGGCCCAGTTGATGA
      361 ---------+---------+---------+---------+---------+---------+ 420
          TGCTTCGGGTCGCGGCCCCGGTCAGCGCGGCCAGCCGGAGGACCAACCGGGTCAACTACT
-7        W  S  A  W  R  R  P  W  D  R  R  D  A  E  Q  N  A  W  N  I   -

          AGTCGAGCACGTCCTCGCGGAACACCGACATCCTGCCGGCCTGGATATTGAAGACGTGGT
      421 ---------+---------+---------+---------+---------+---------+ 480
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-7        F  D  L  V  D  E  R  F  V  S  M  R  G  A  Q  I  N  F  V  H   -

          CCCAGGGGTTGCCGTCACGGTGATAGGCGACGCCGGCCGAGCGGTAgGCGGCGCGCCGCT
      481 ---------+---------+---------+---------+---------+---------+ 540
          GGGTCCCCAACGGCAGTGCCACTATCCGCTGCGGCCGGCTCGCCATcCGCCGCGCGGCGA
-7        D  W  P  N  G  D  R  H  Y  A  V  G  A  S  R  Y  A  A  R  R   -

          CCAGGAGGACGACTTCCAGCGGTCTTCTCGCGAAATGAAGCAGGCGTATCGCGGTCGCCG
      541 ---------+---------+---------+---------+---------+---------+ 600
          GGTCCTCCTGCTGAAGGTCGCCAGAAGAGCGCTTTACTTCGTCCGCATAGCGCCAGCGGC
-7        E  L  L  V  V  E  L  P  R  R  A  F  H  L  L  R  I  A  T  A   -

          TGCCTGCCAGGCCCGCCCCTACGACCAGCACCCTGGGGCGCGCACCCGTCATGCCCATGA
      601 ---------+---------+---------+---------+---------+---------+ 660
          ACGGACGGTCCGGGCGGGGATGCTGGTCGTGGGACCCCGCGCGTGGGCAGTACGGGTACT
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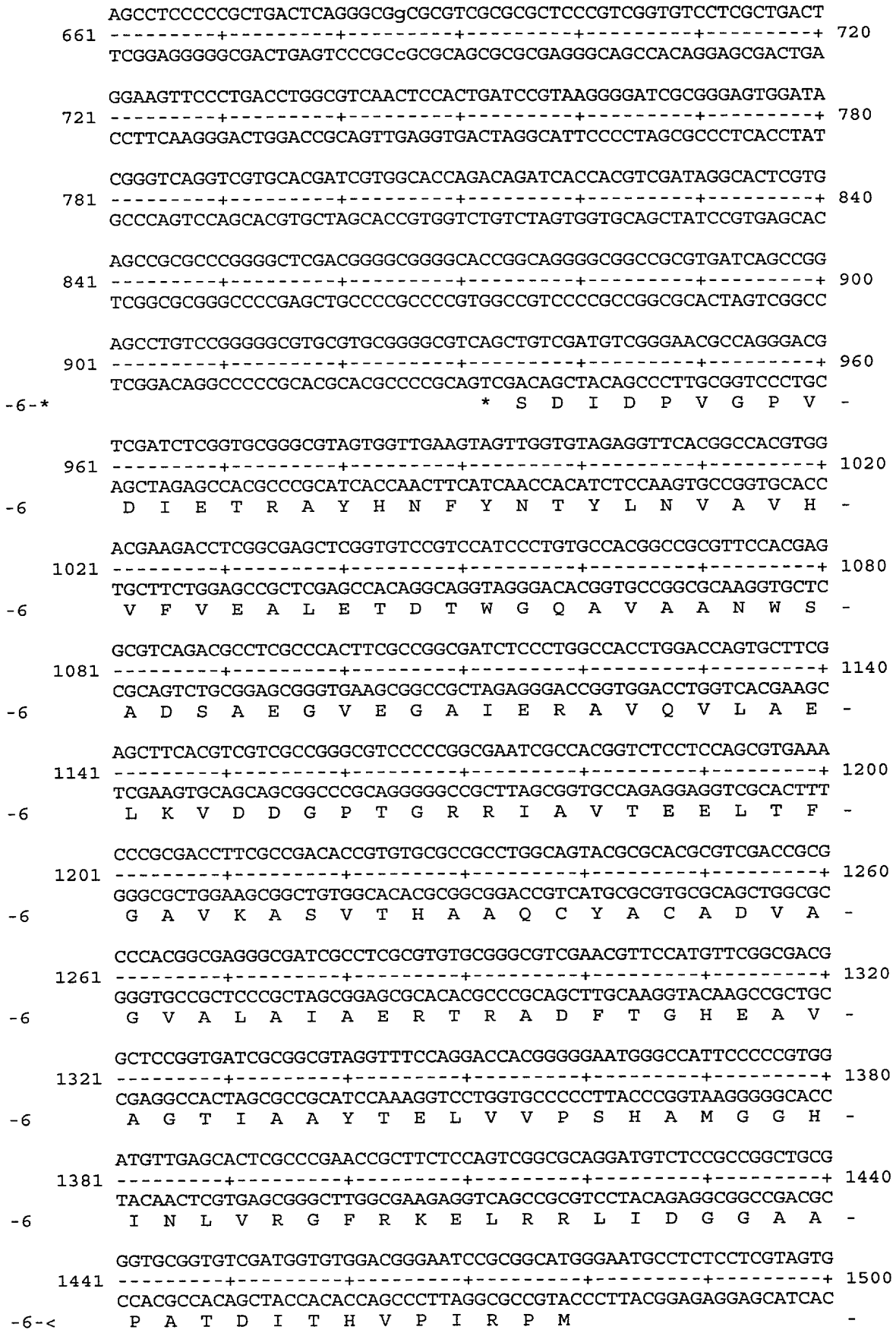

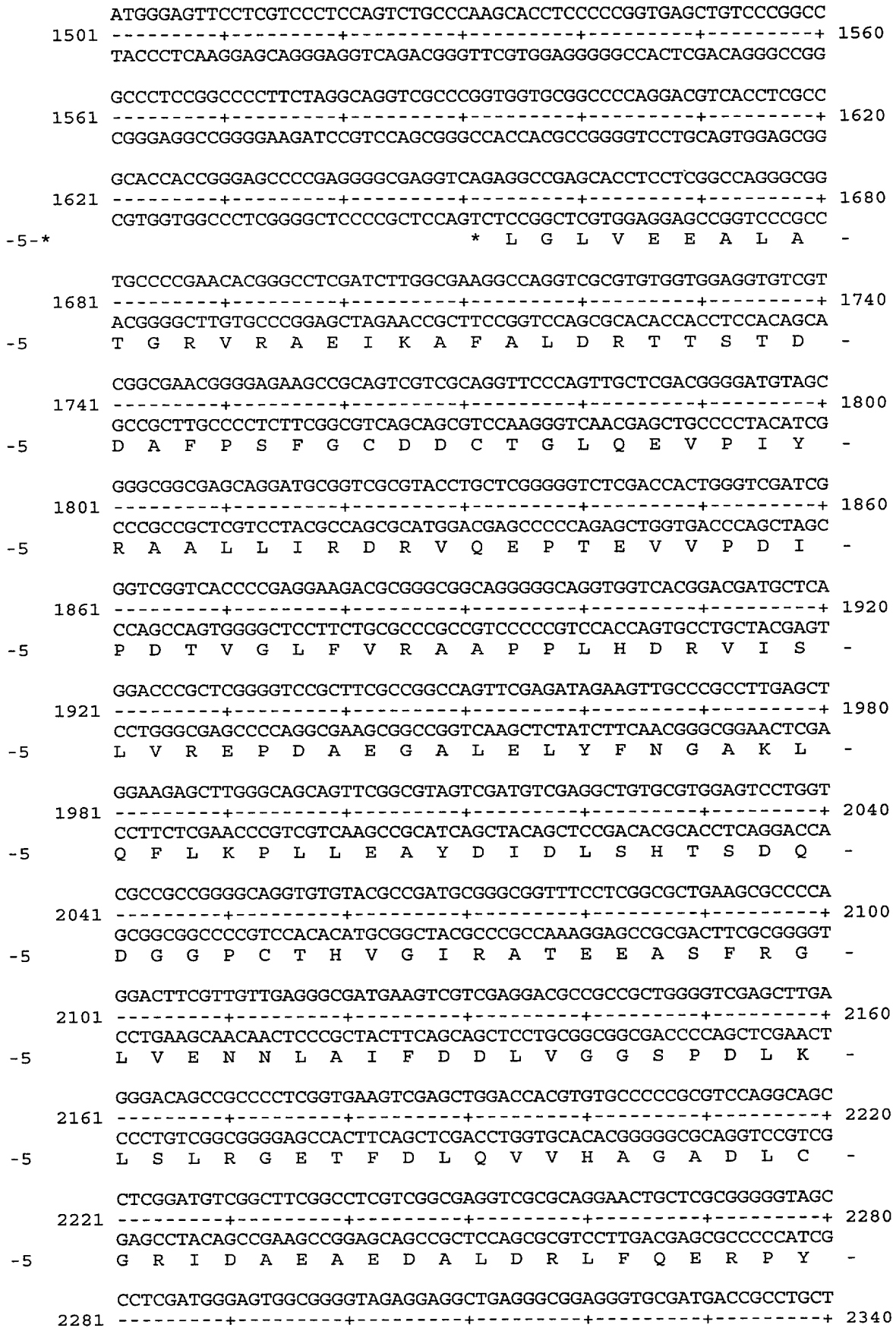

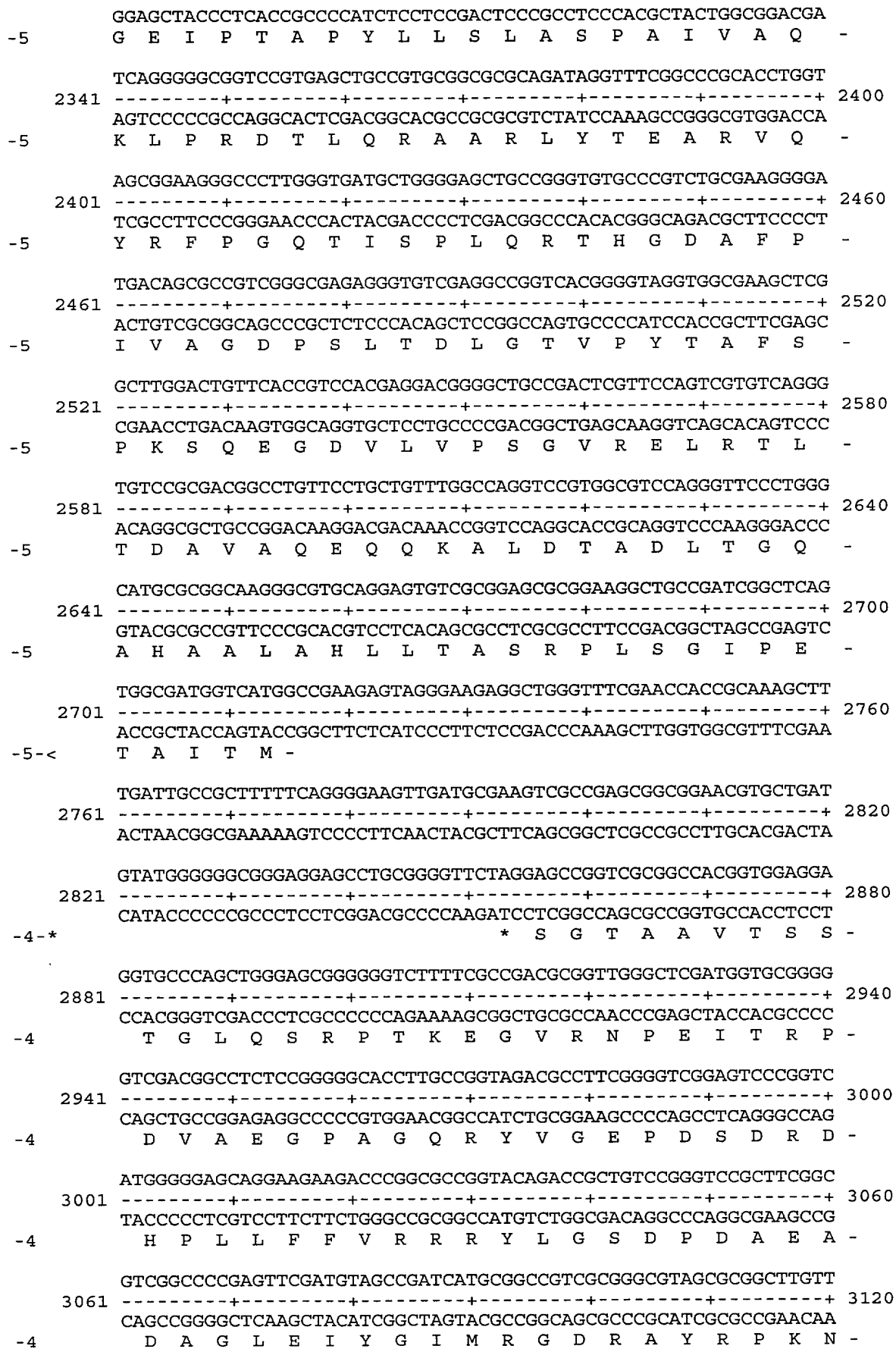

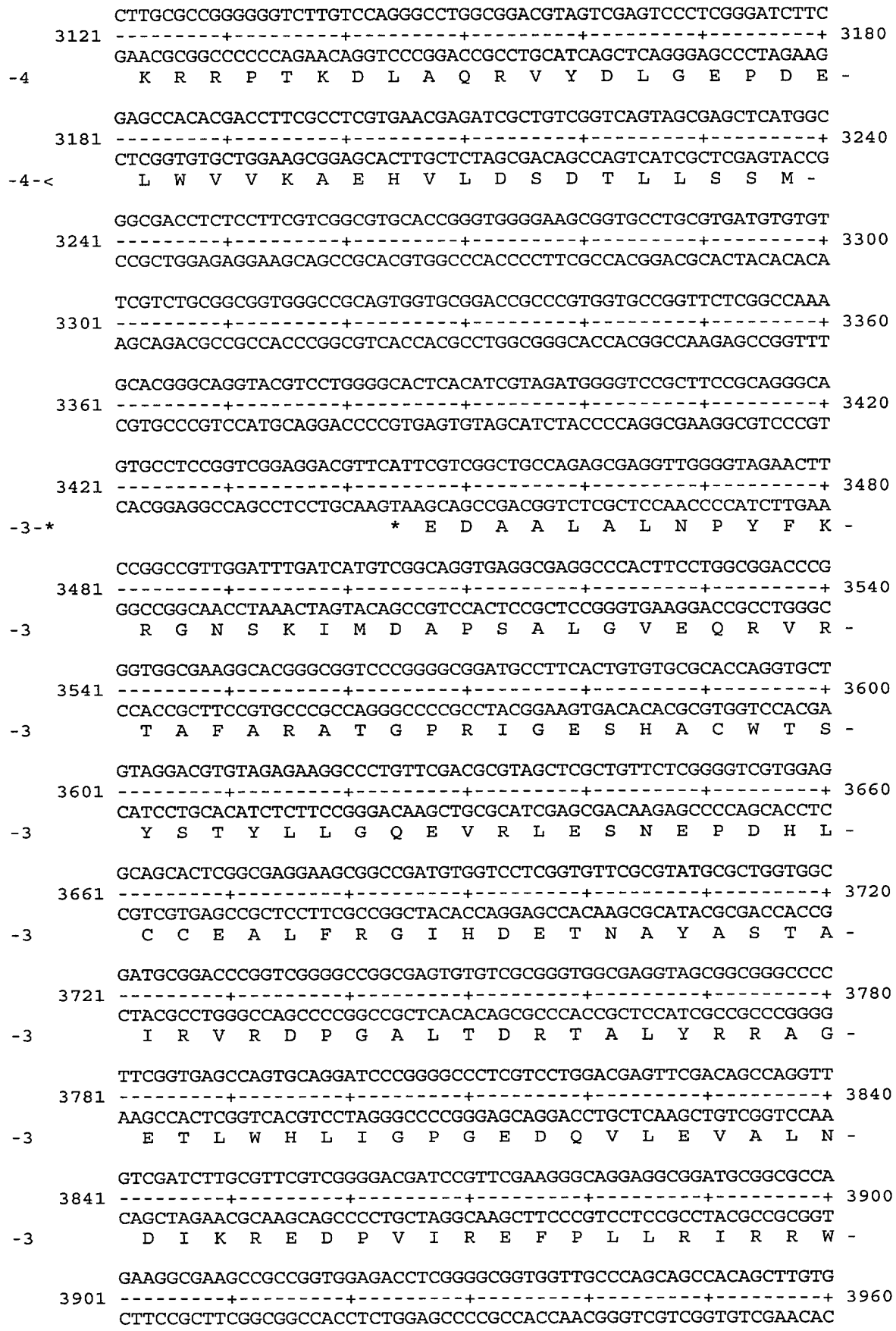

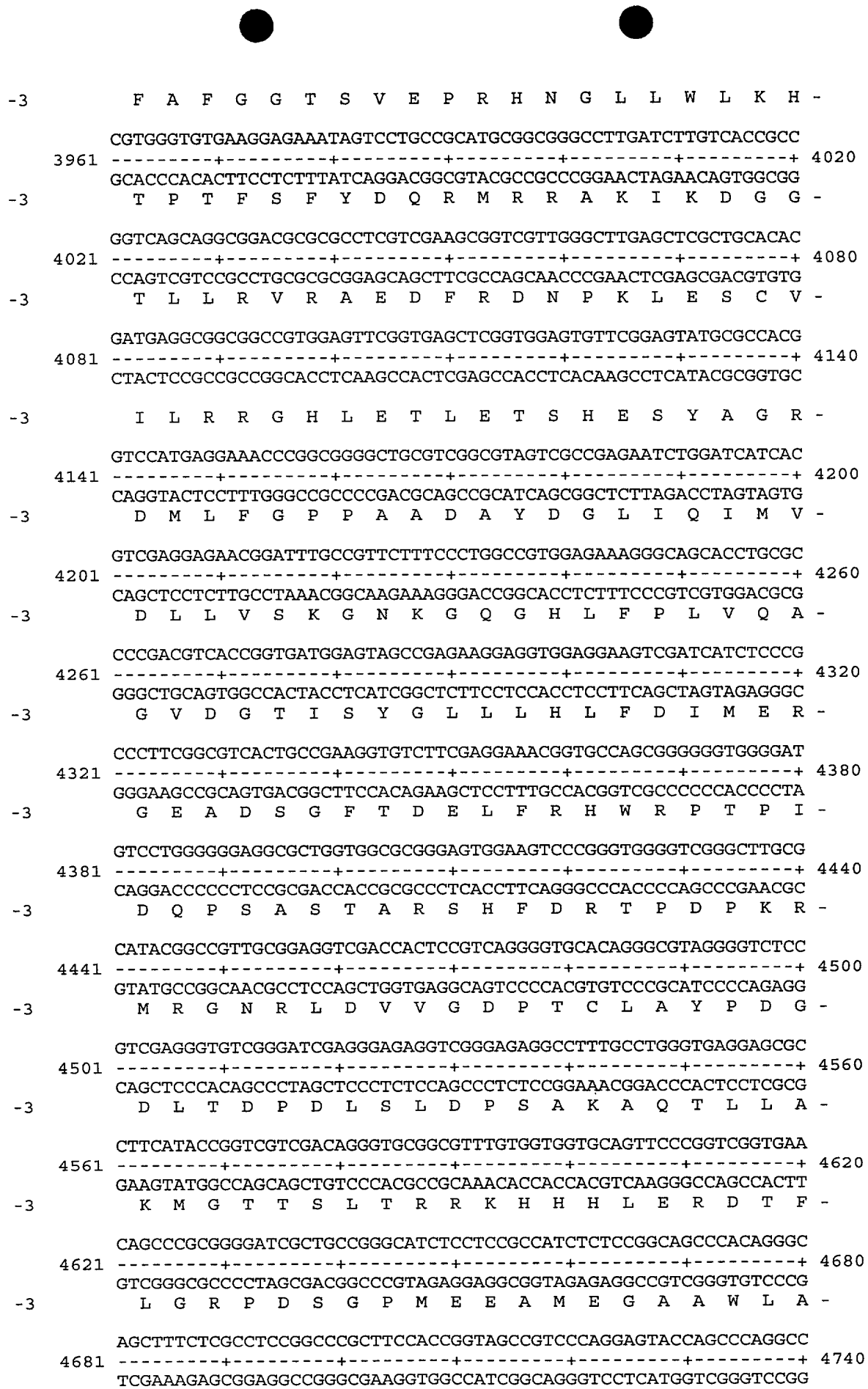
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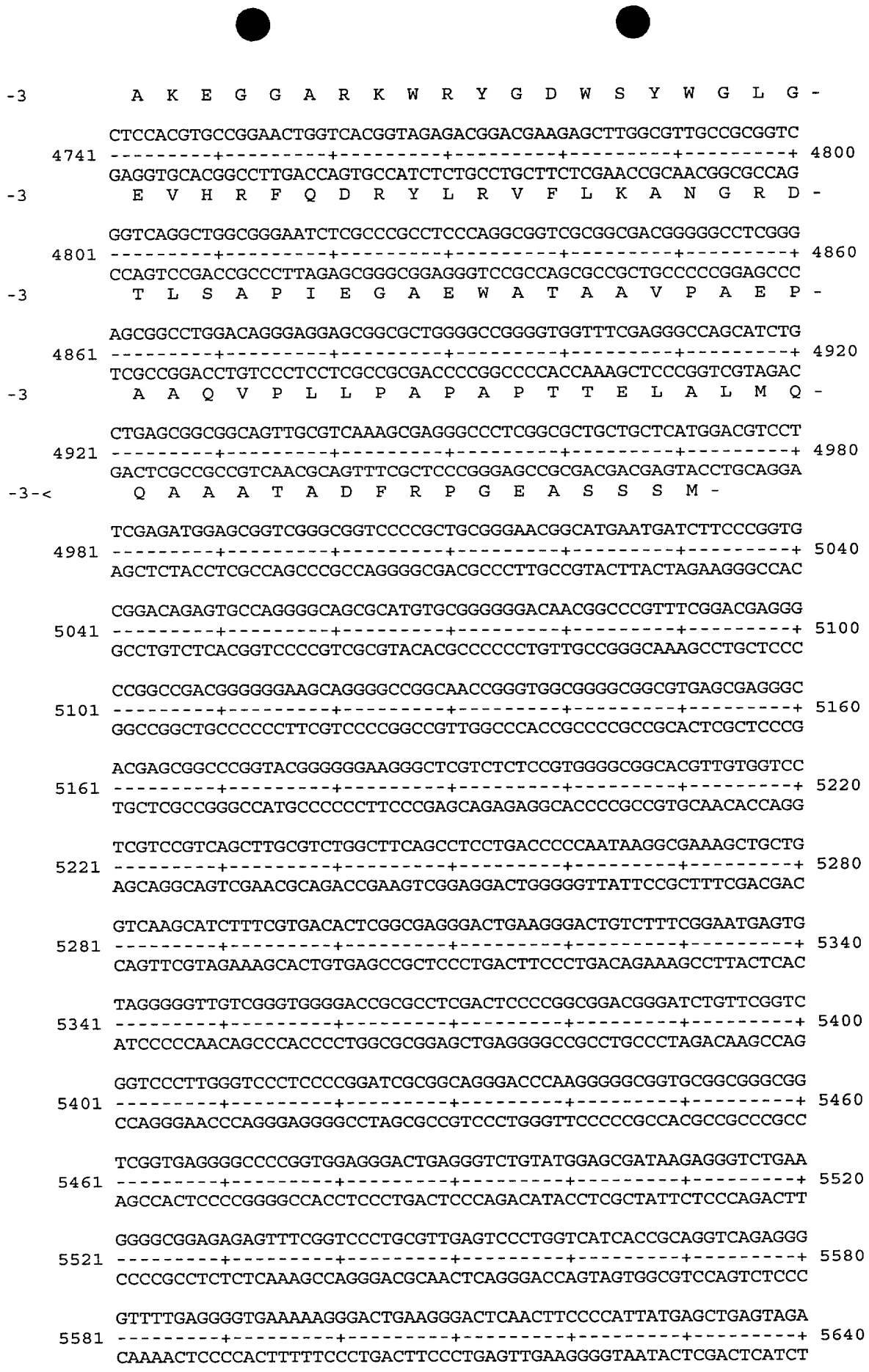

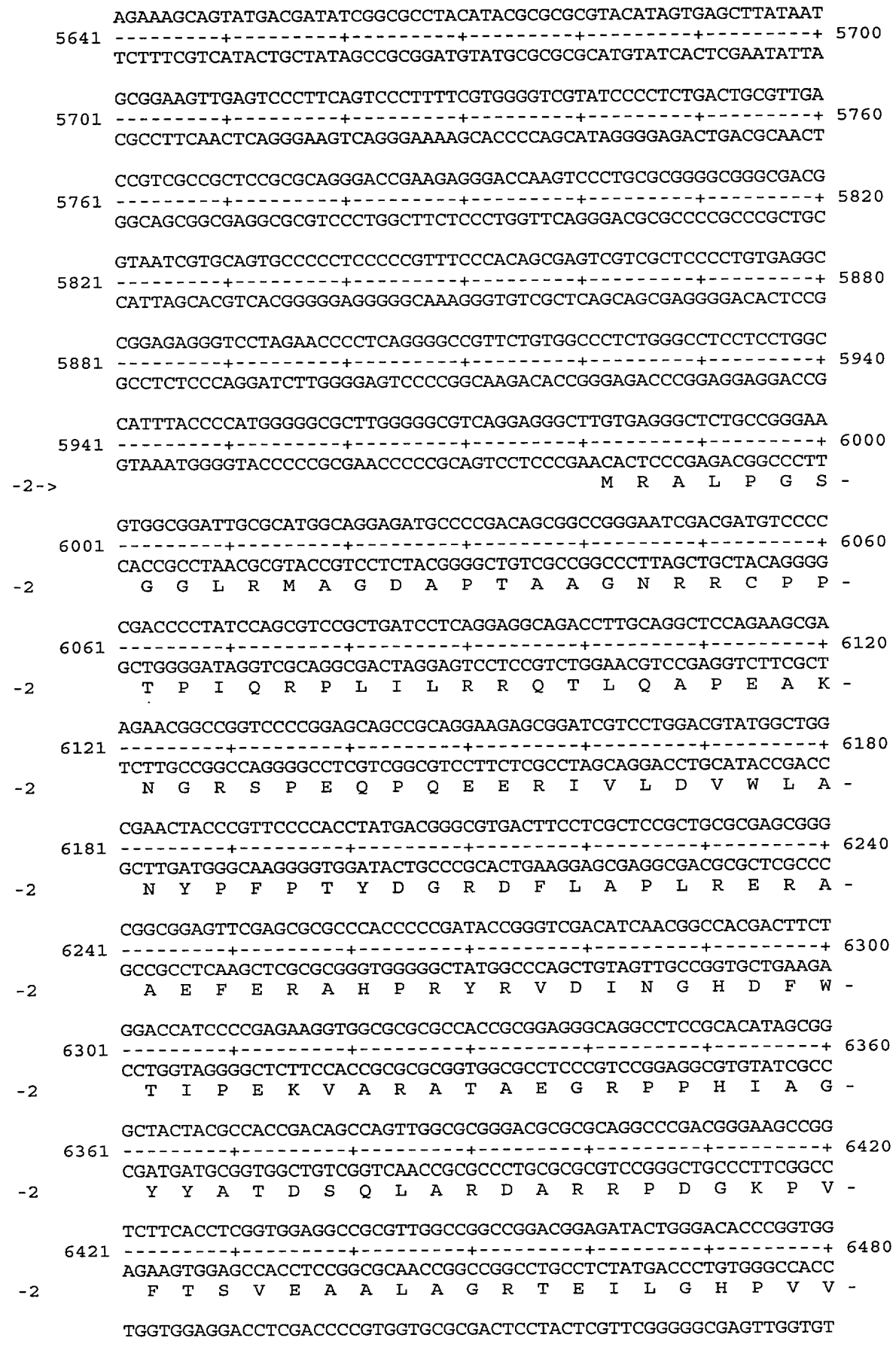
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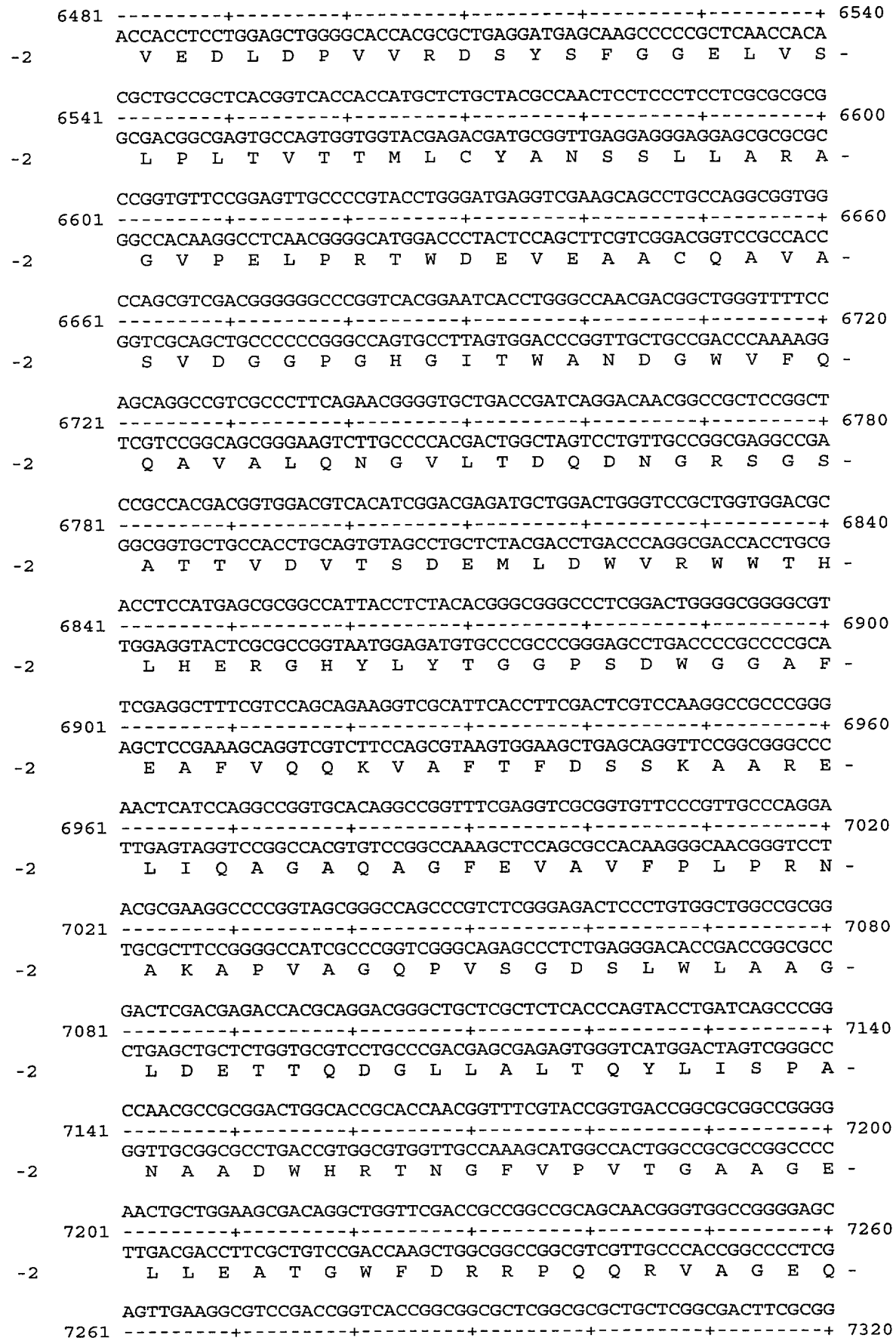
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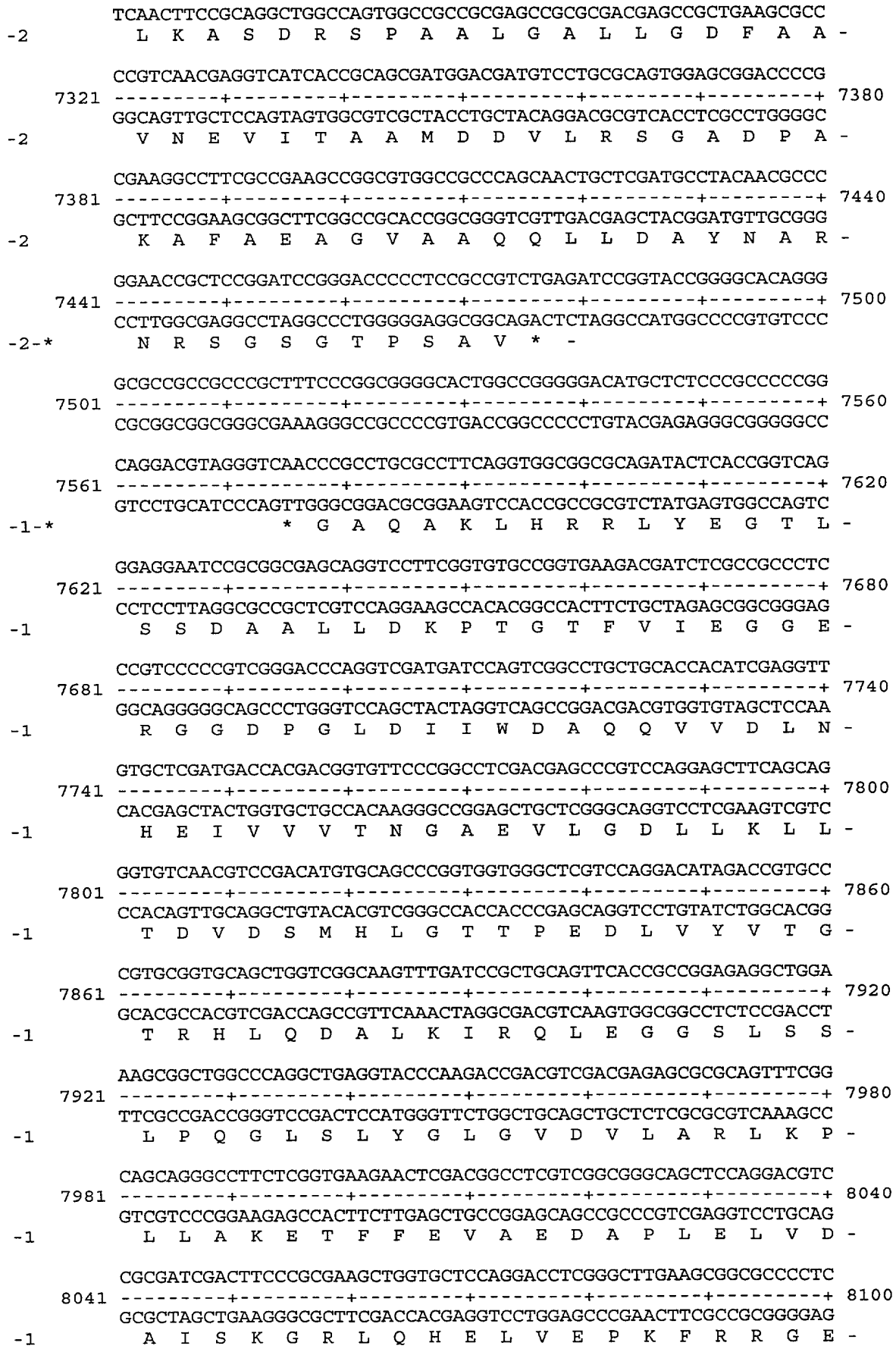

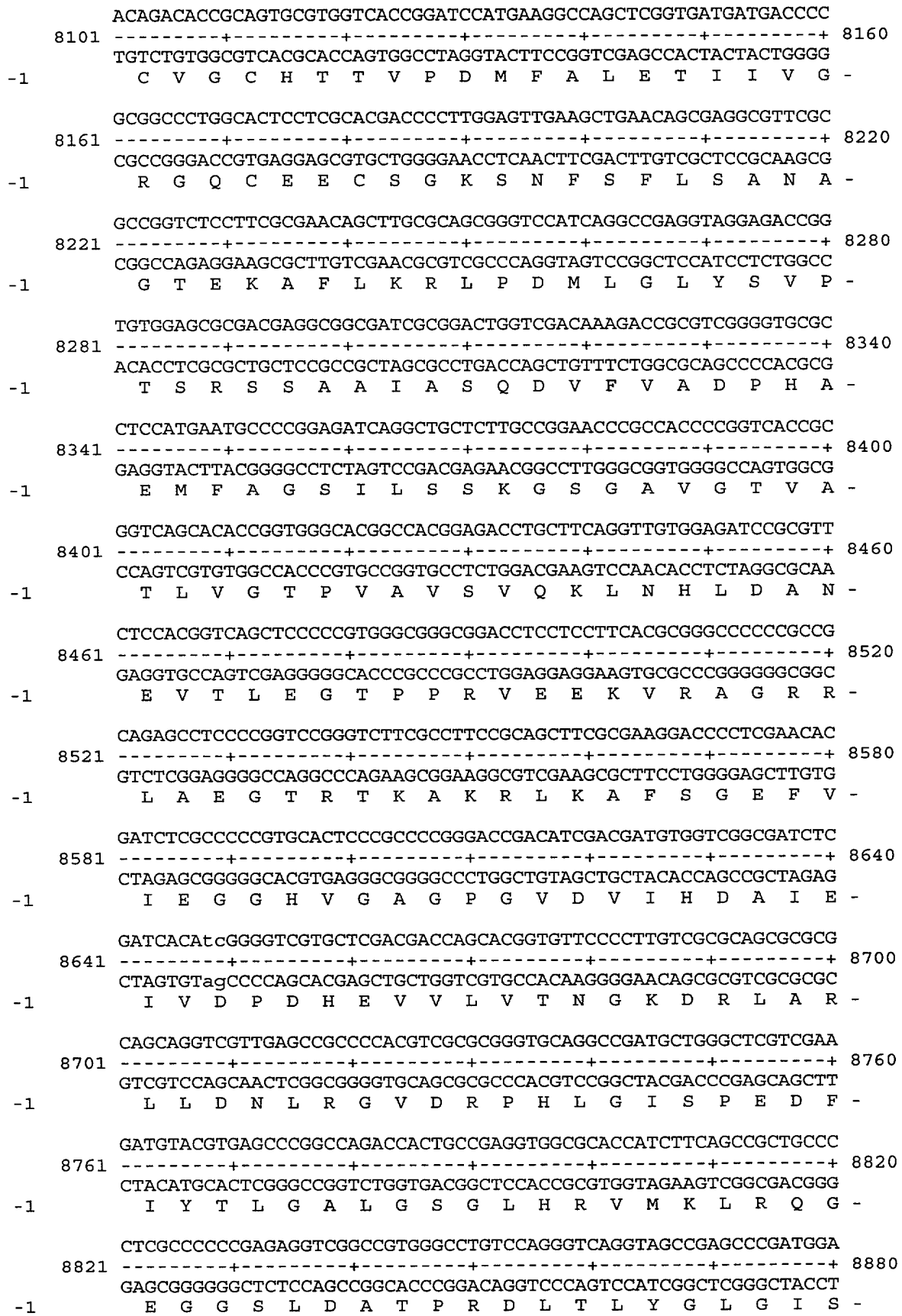

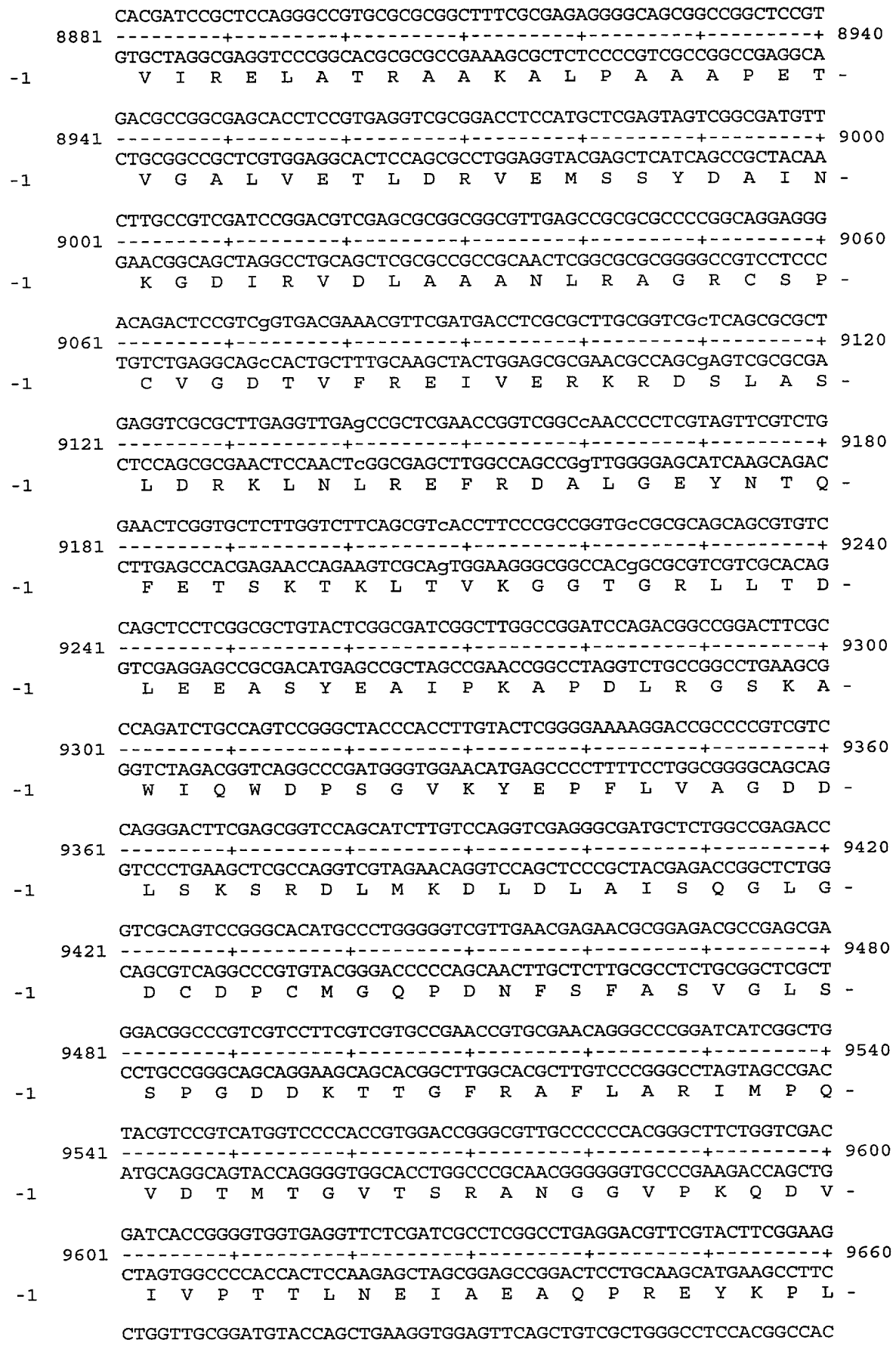
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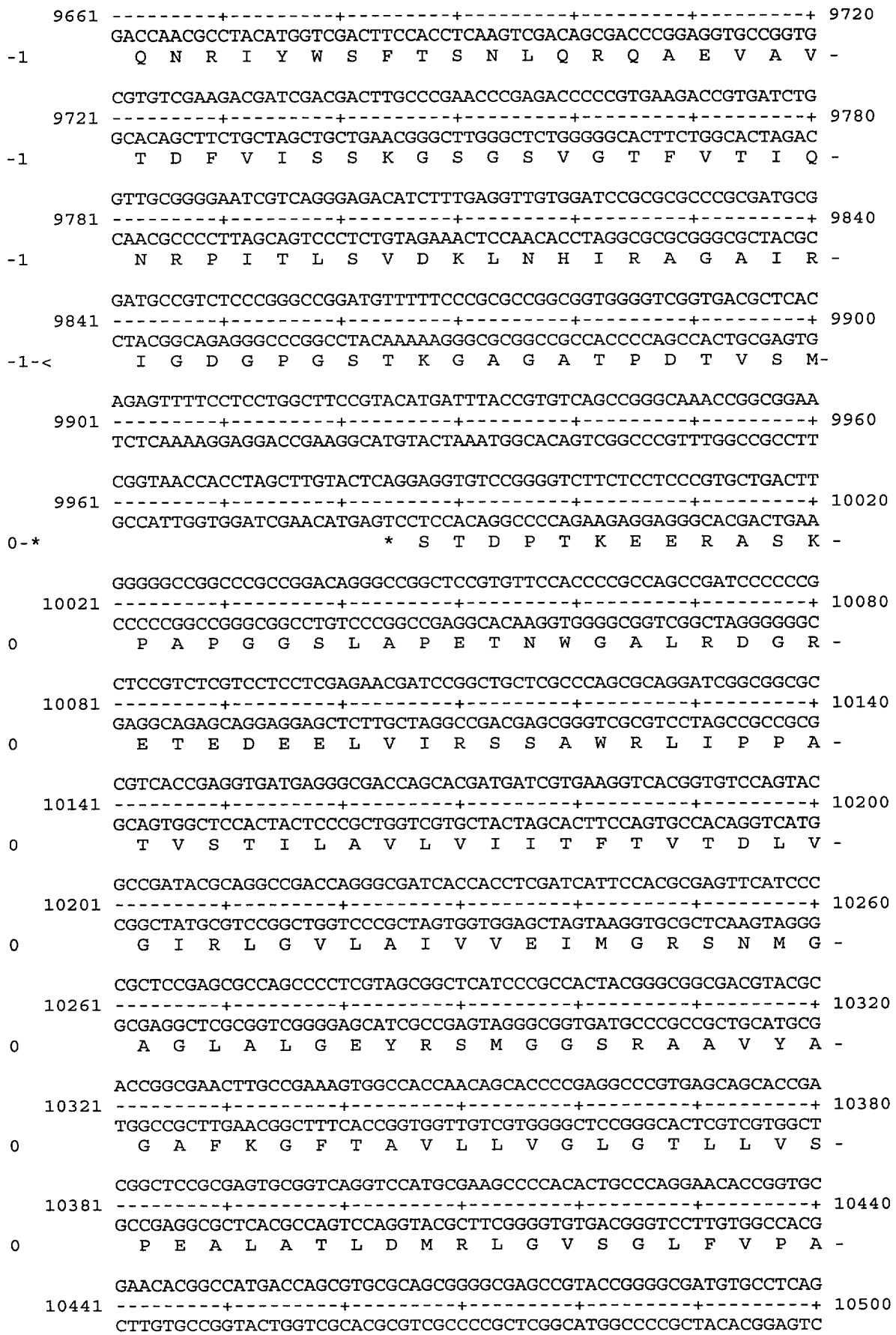

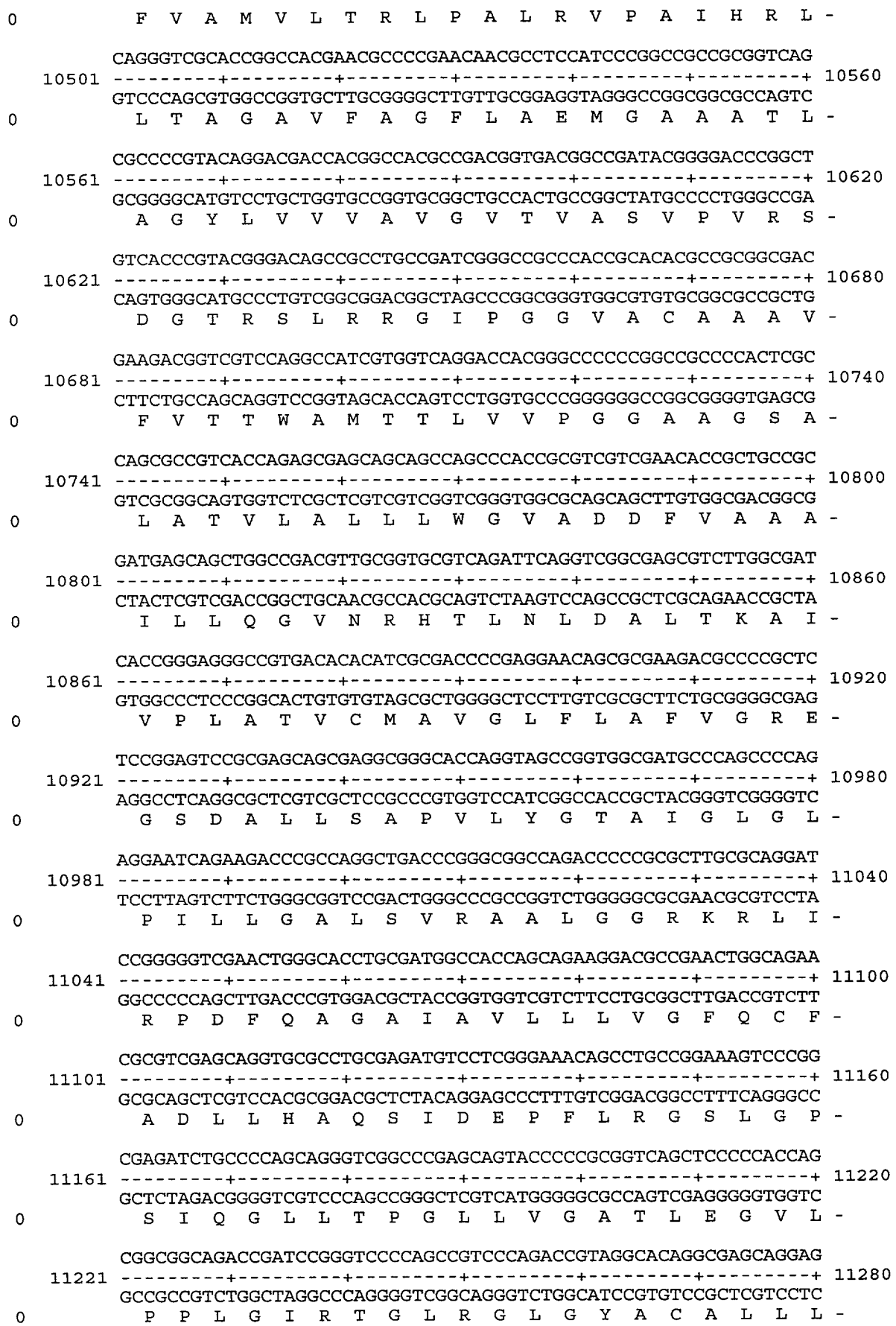
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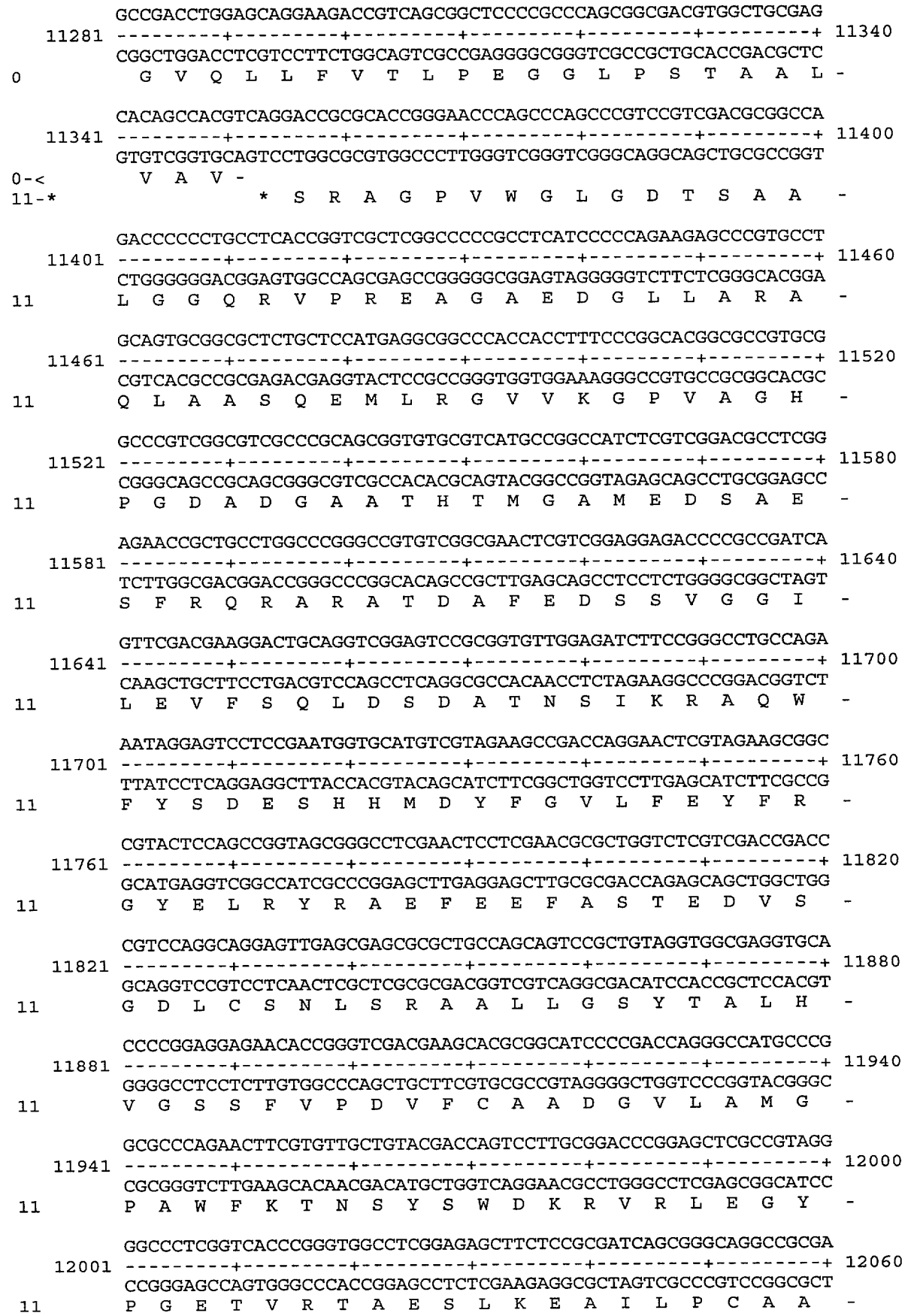
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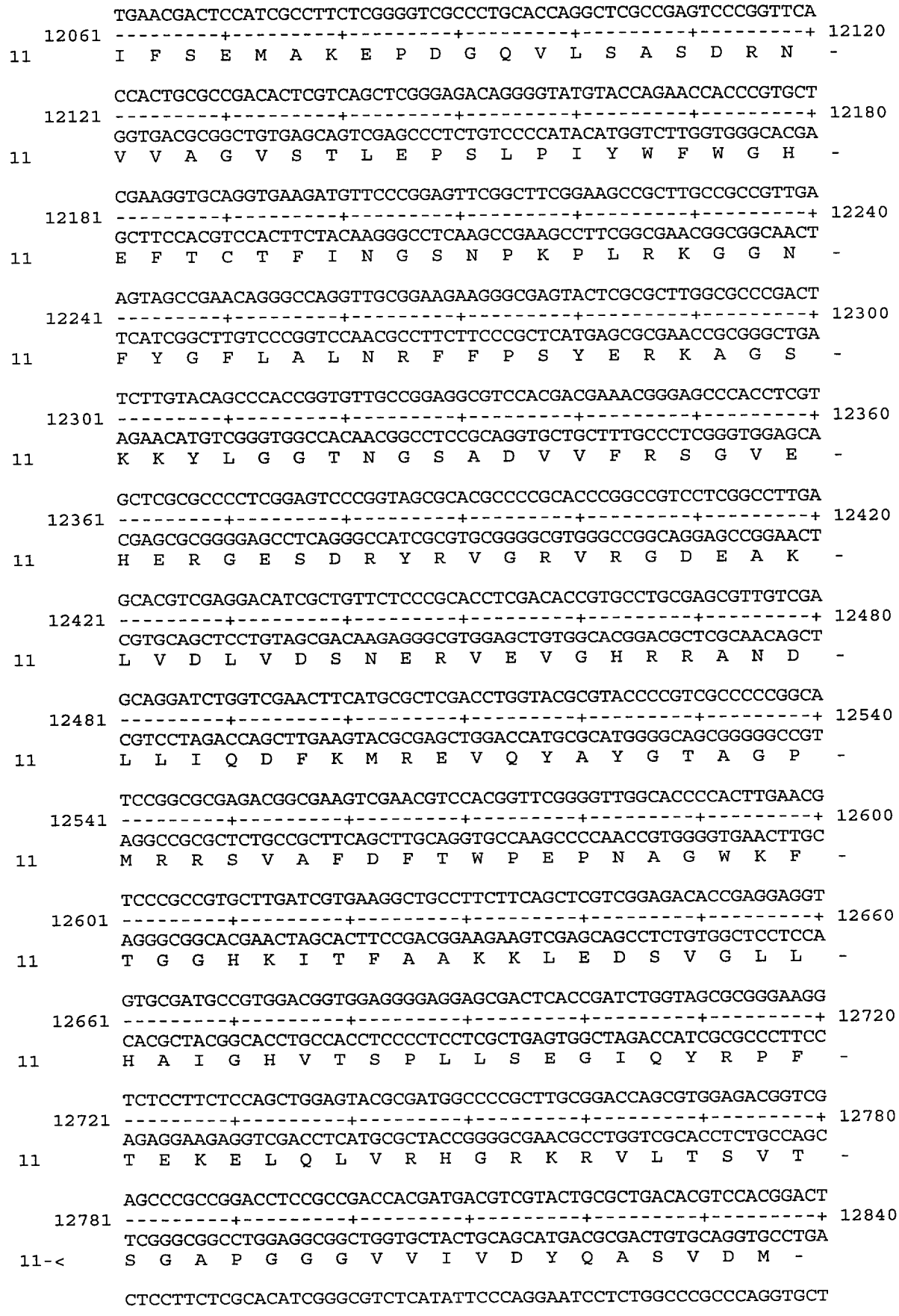

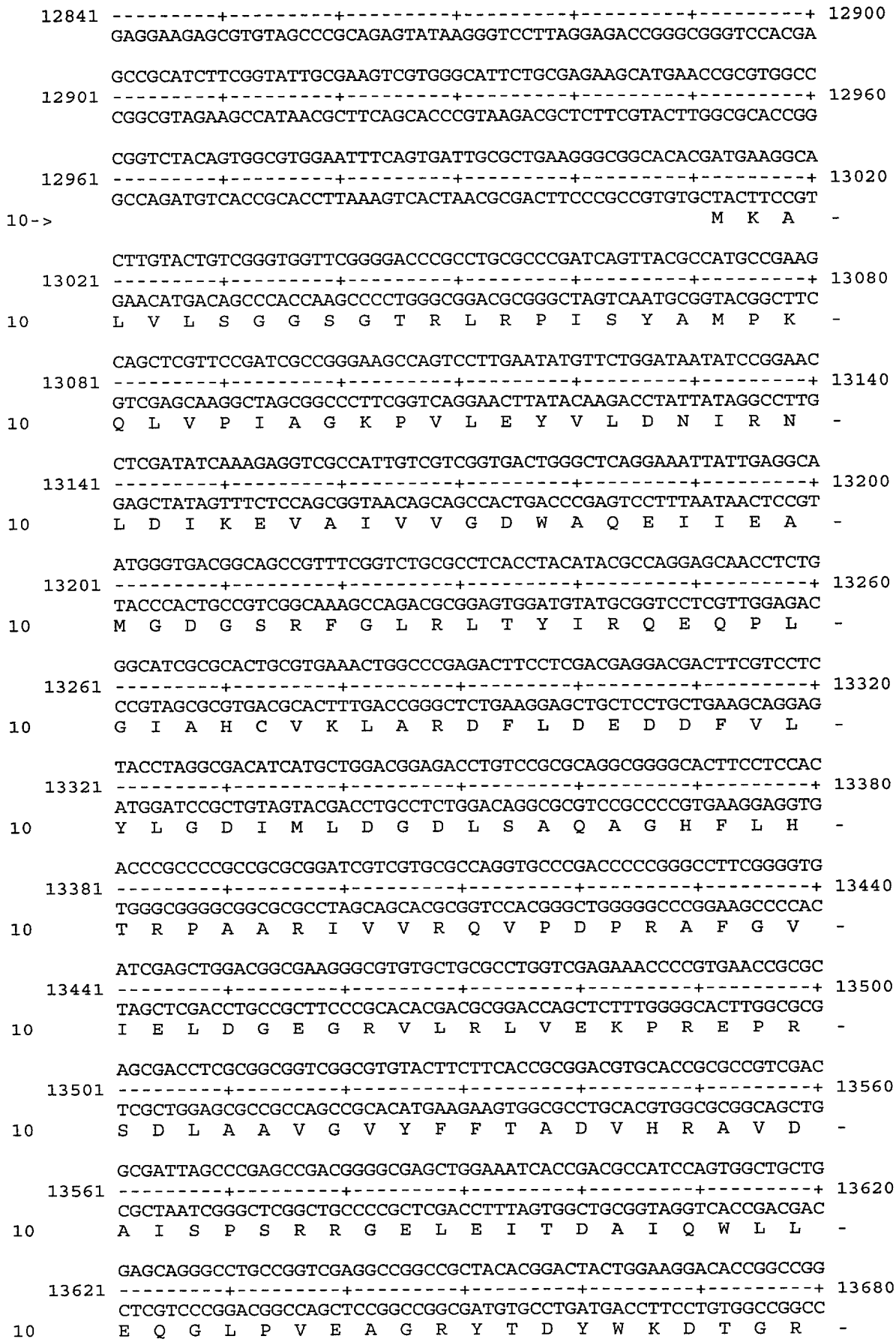
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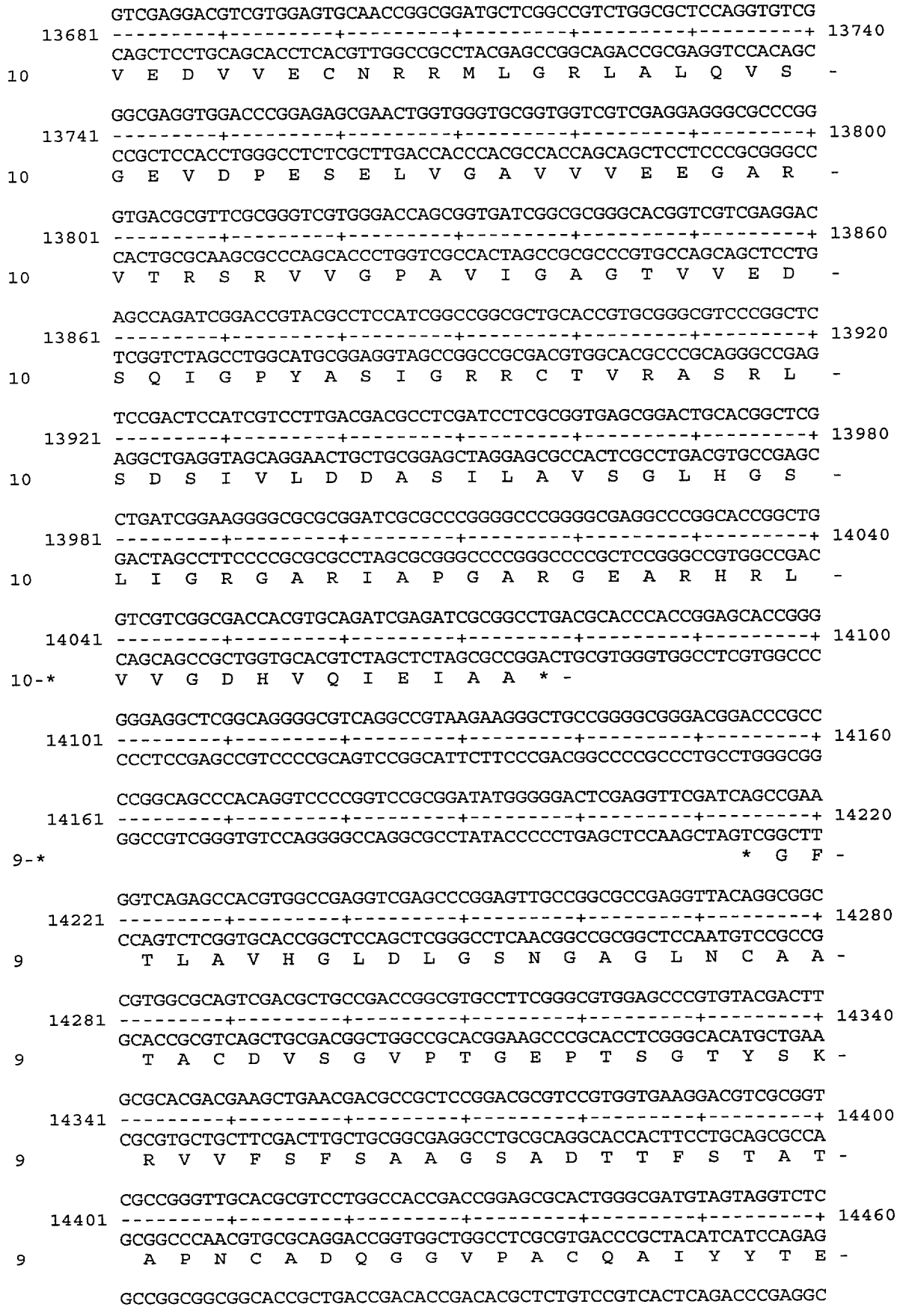
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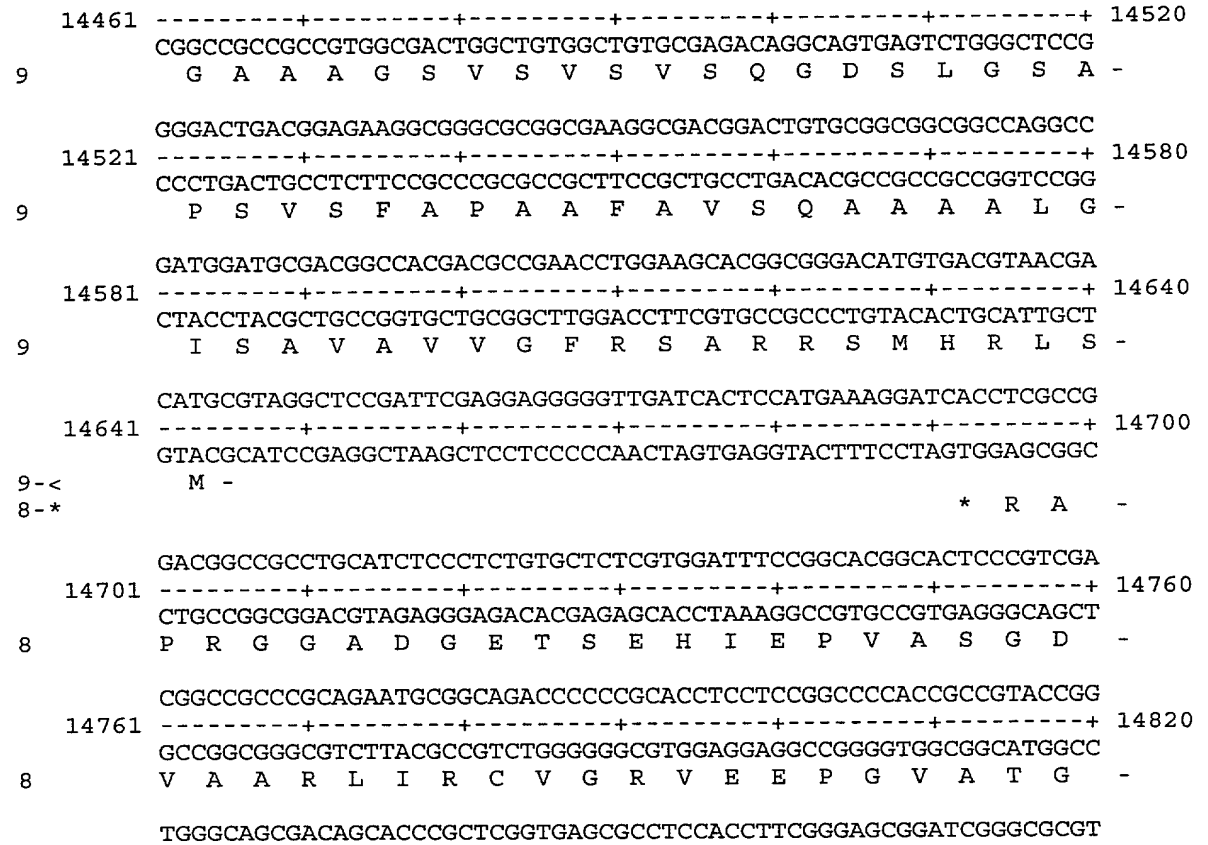

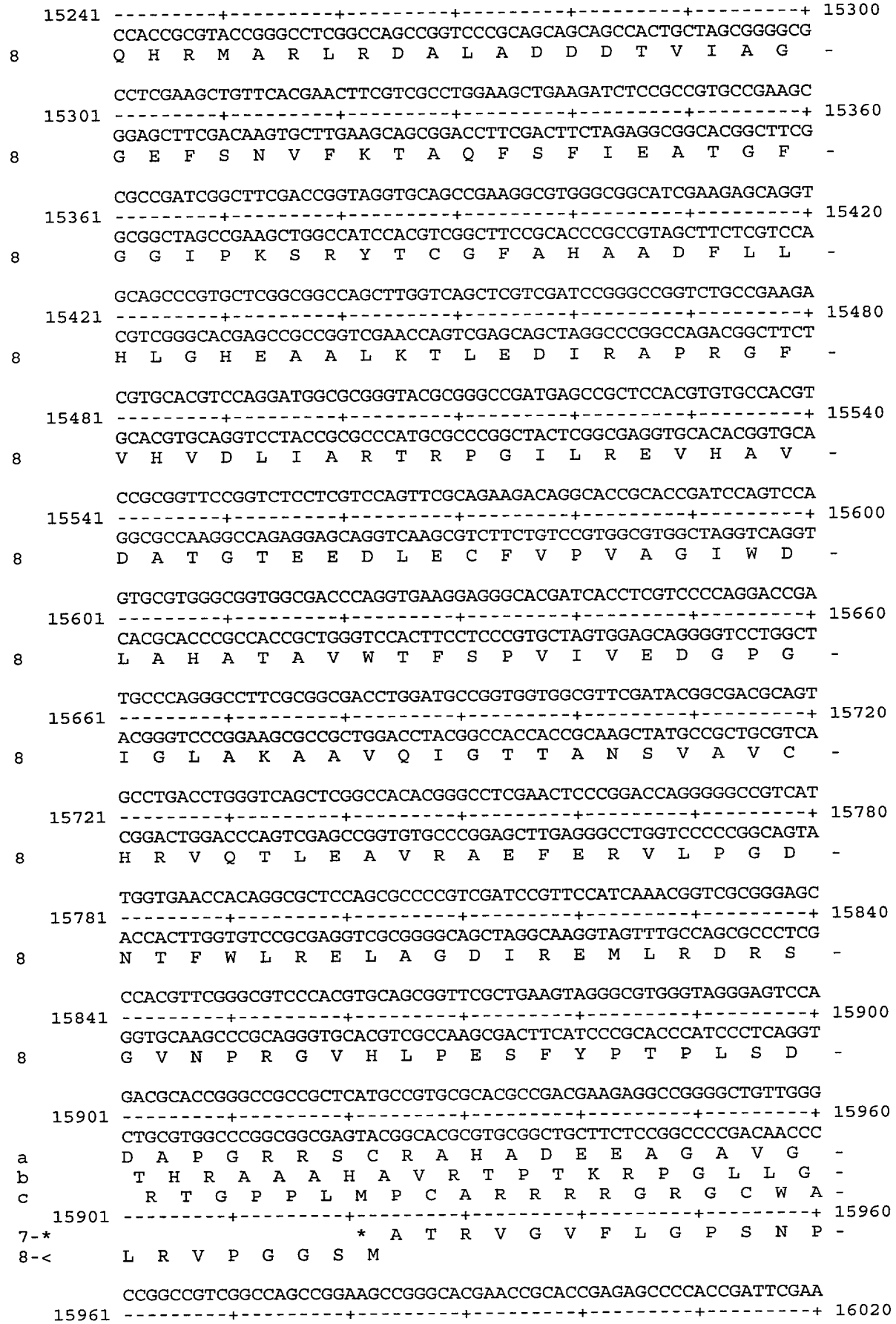
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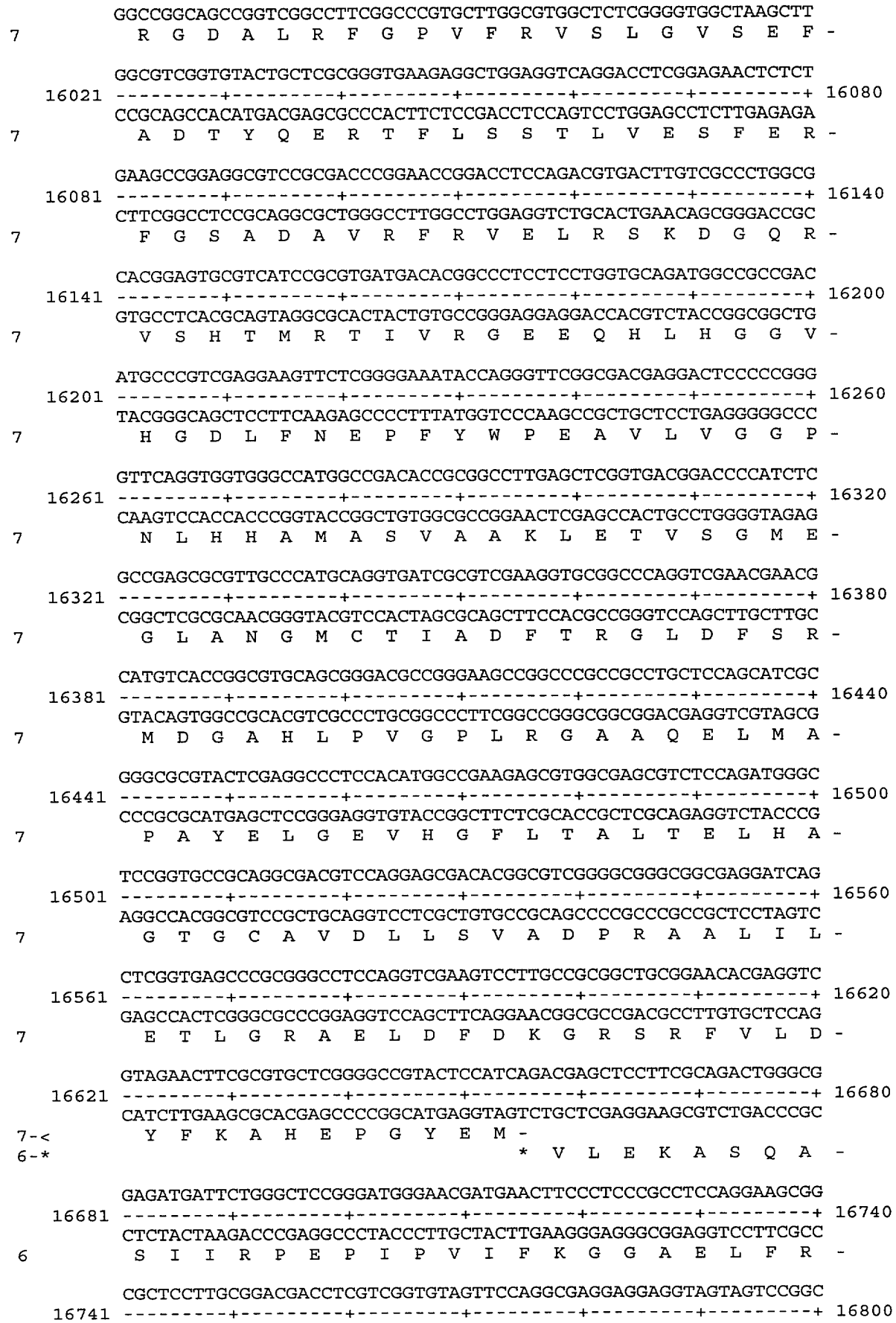
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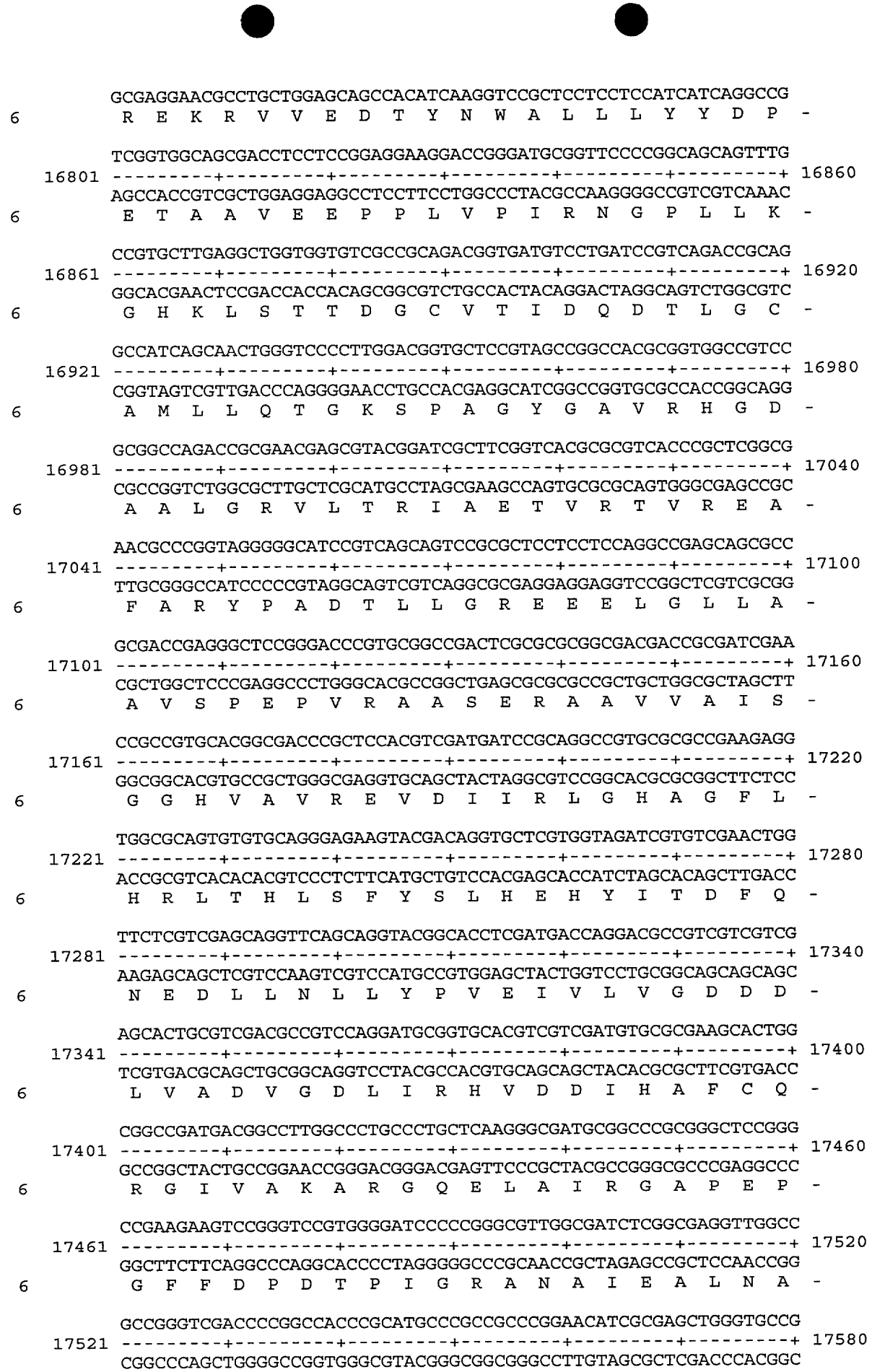
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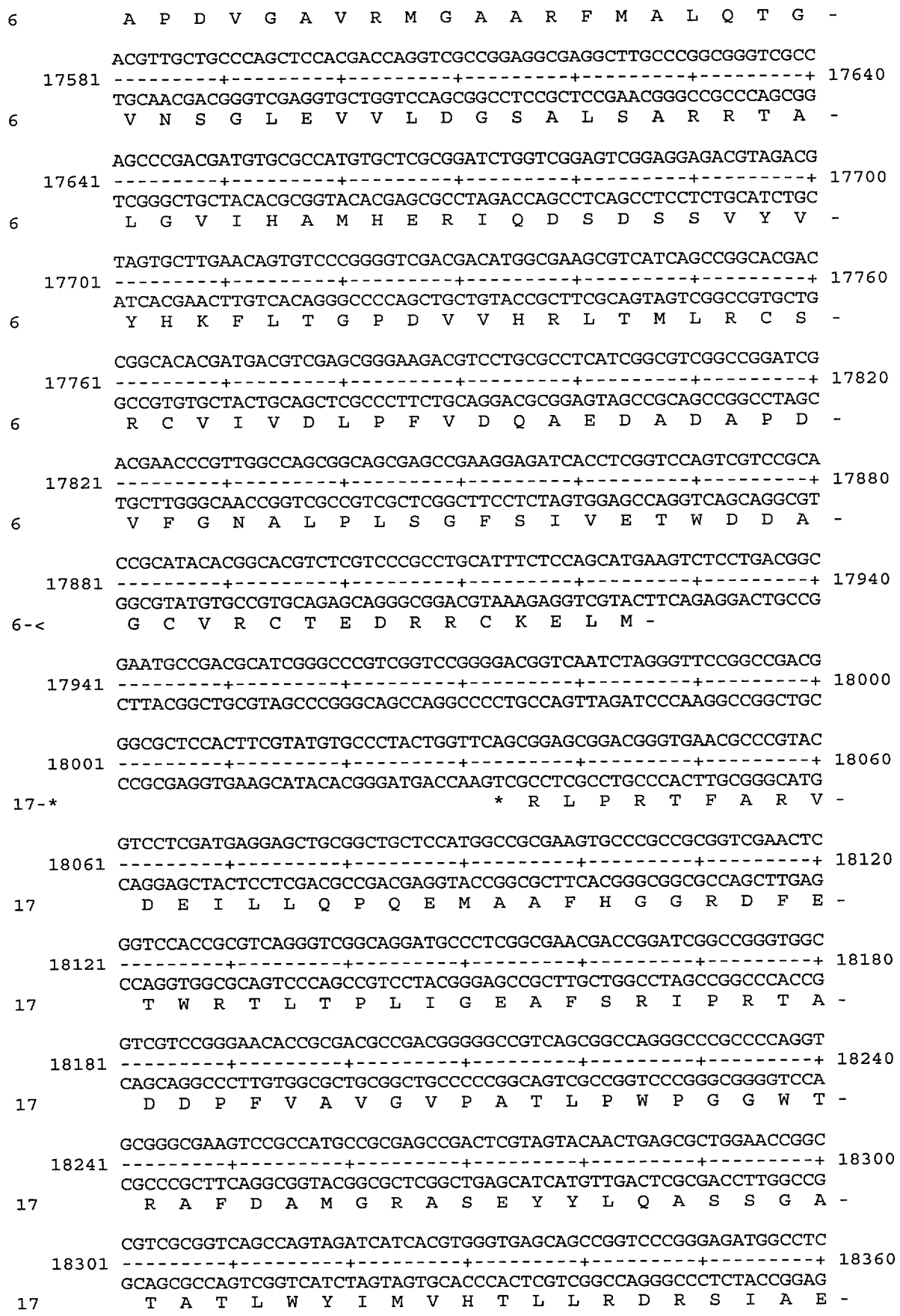
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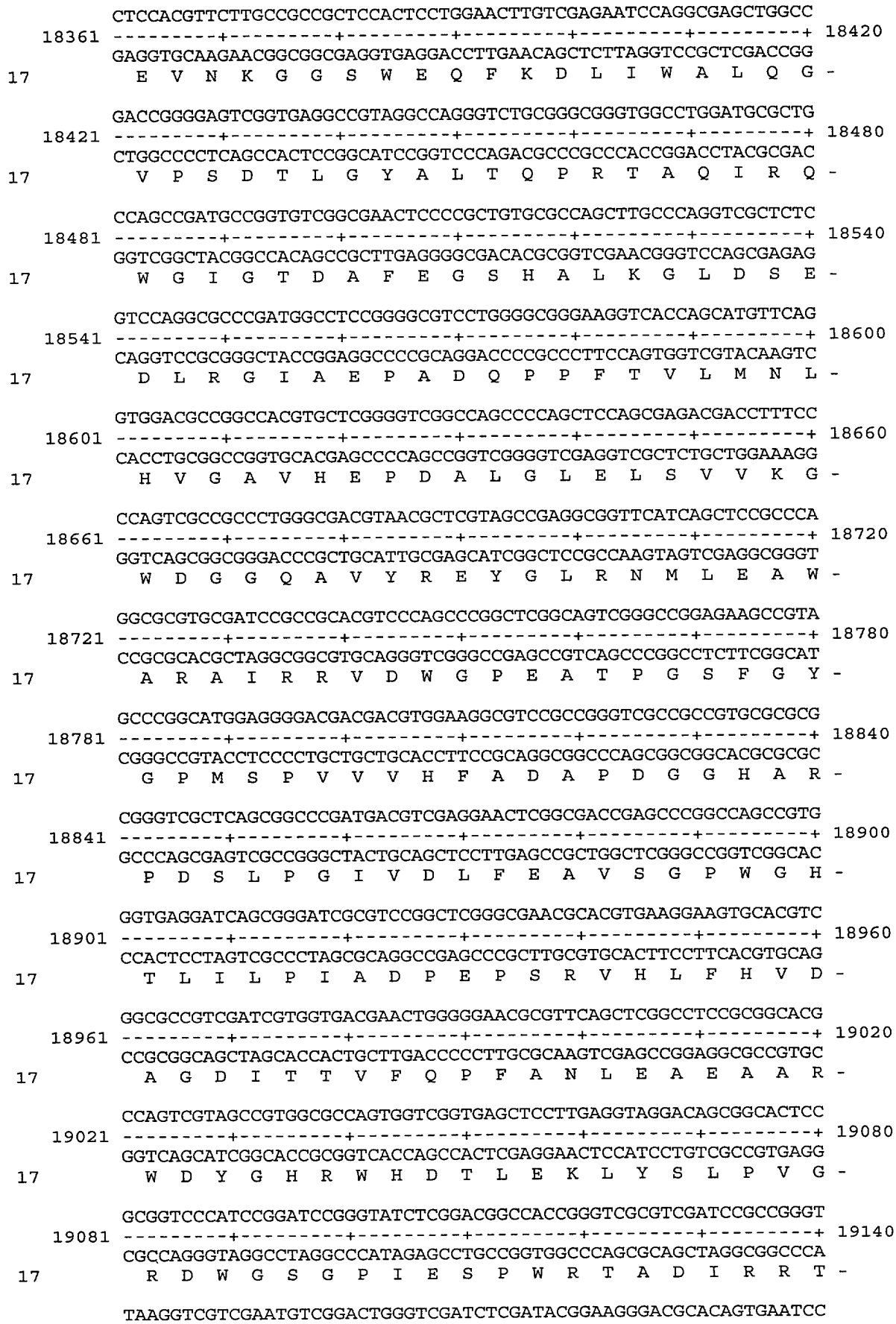
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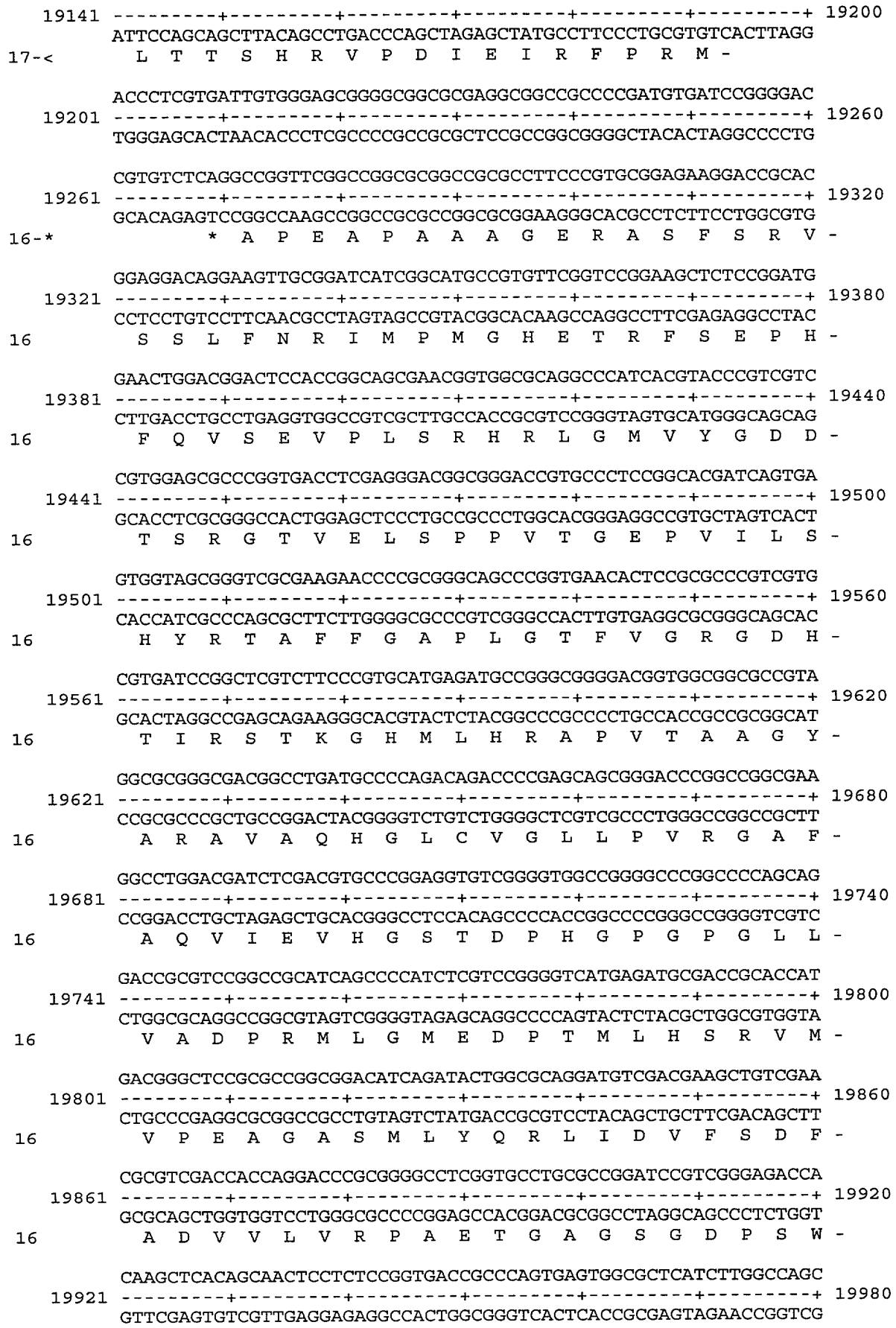

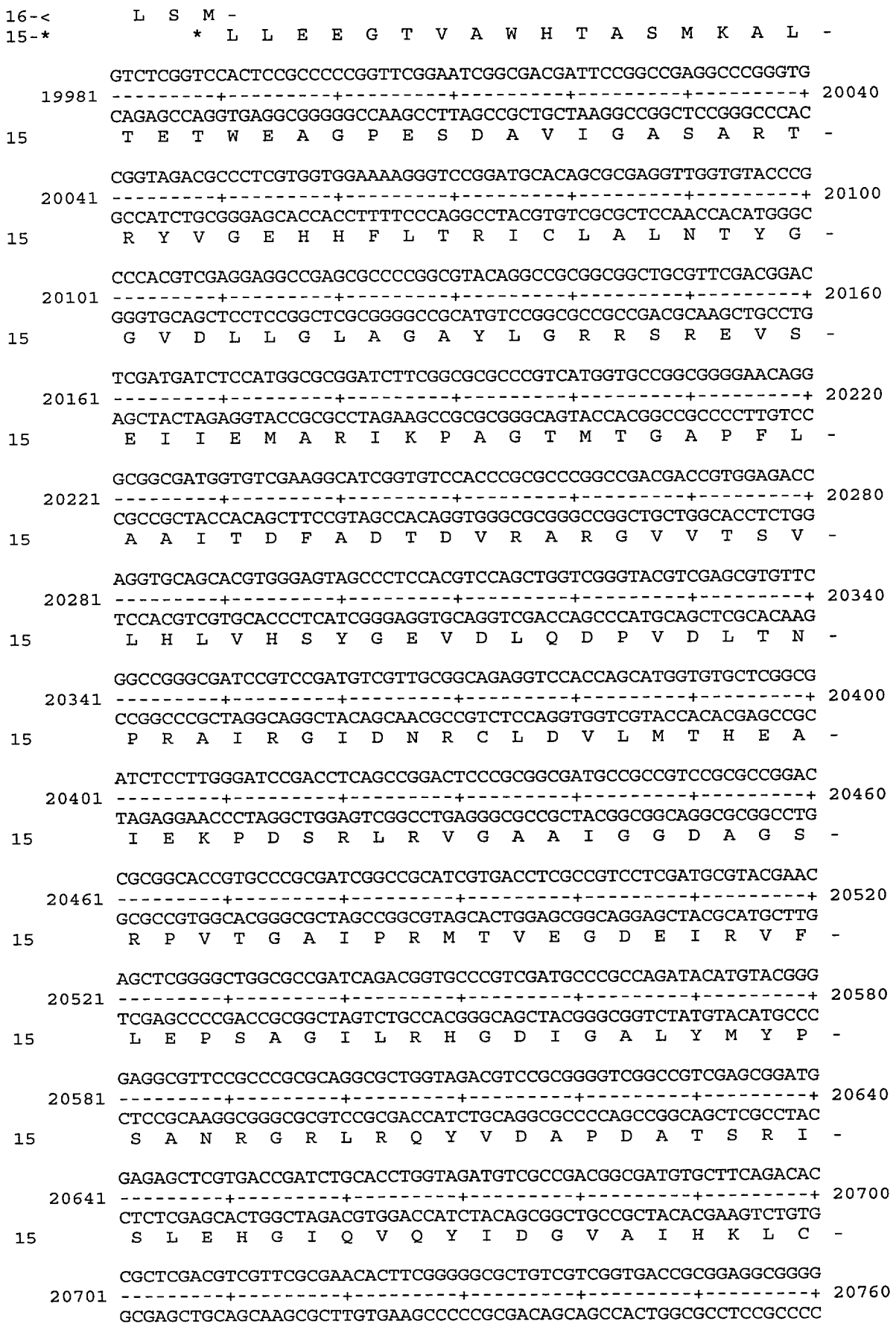

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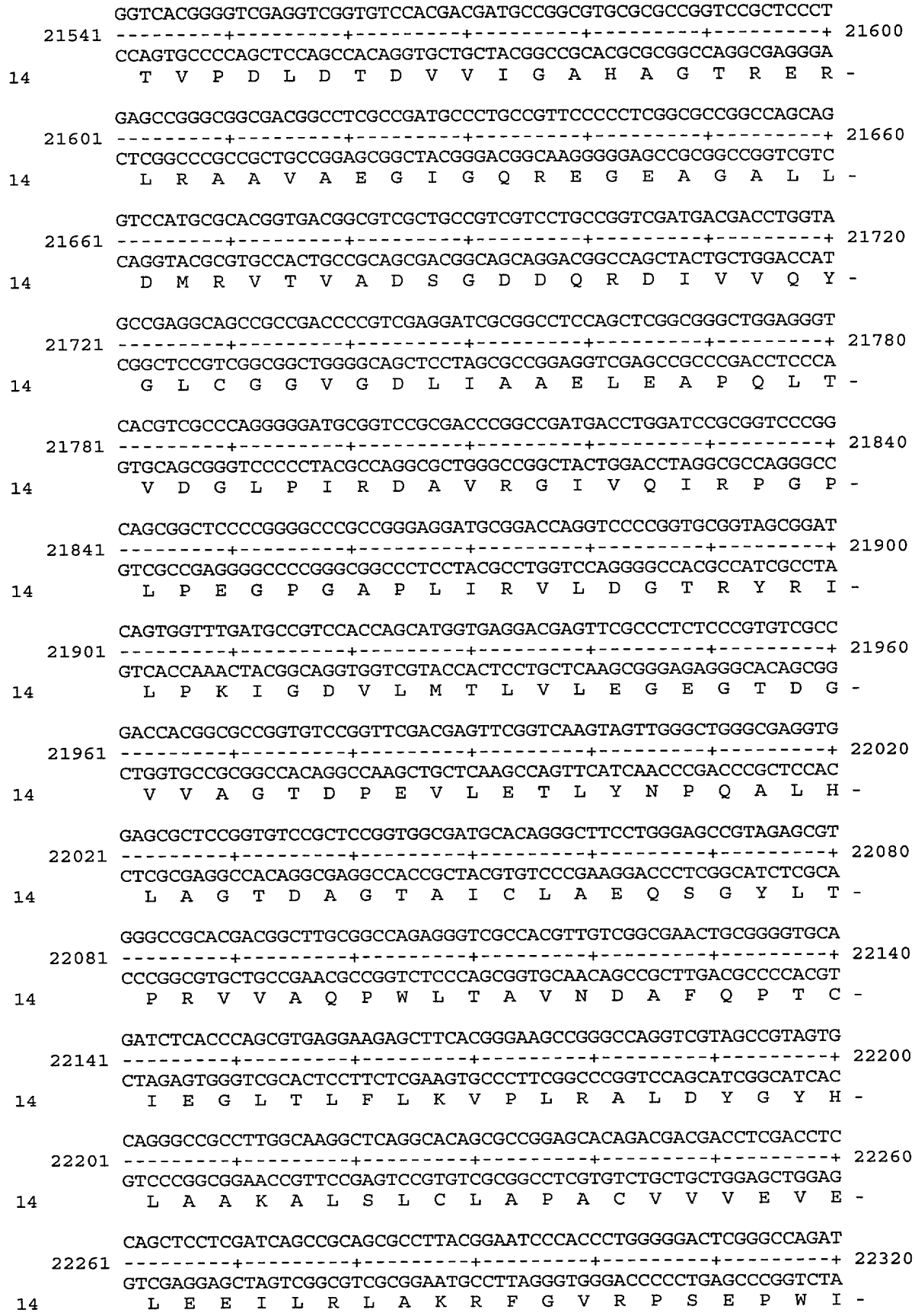

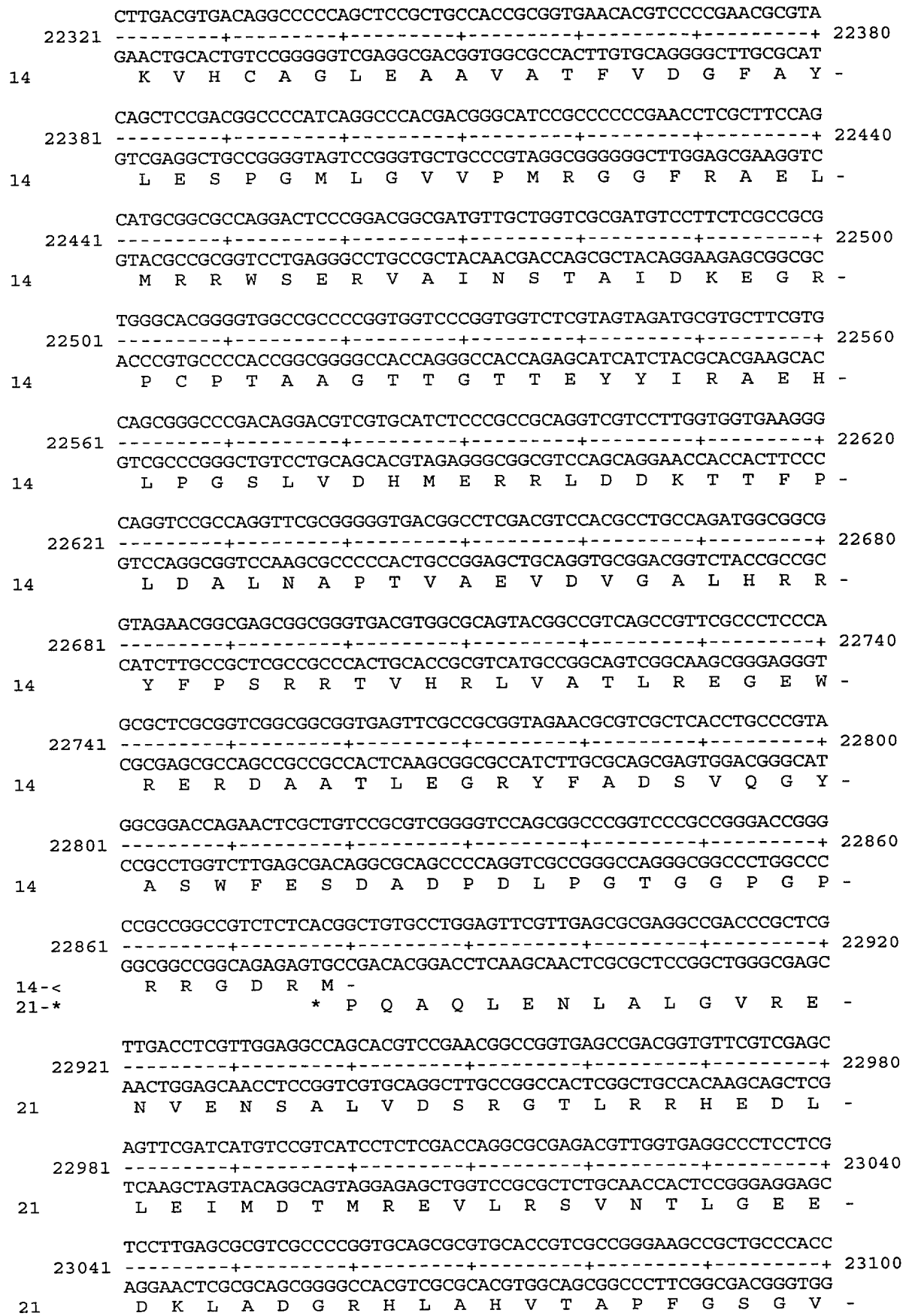
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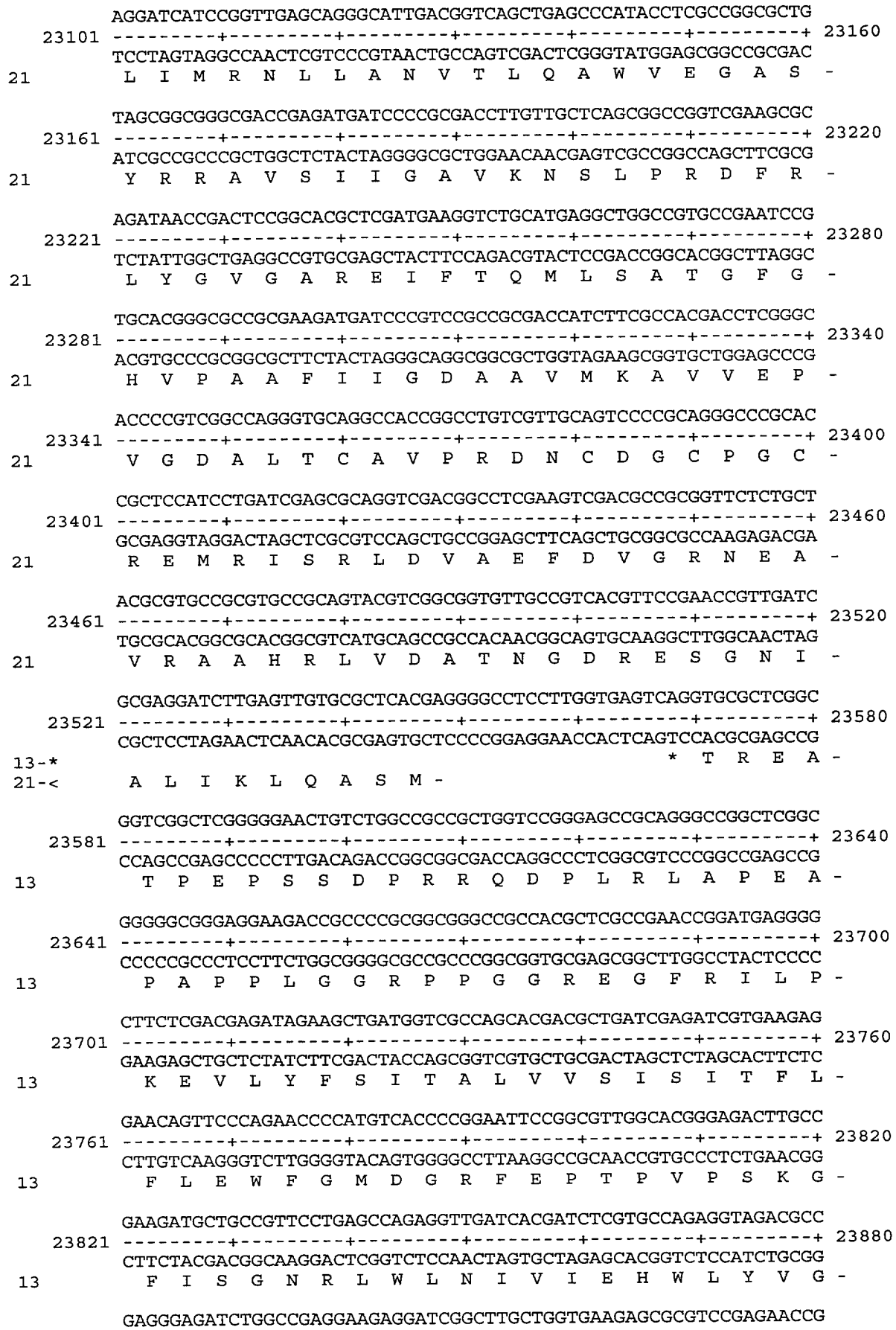
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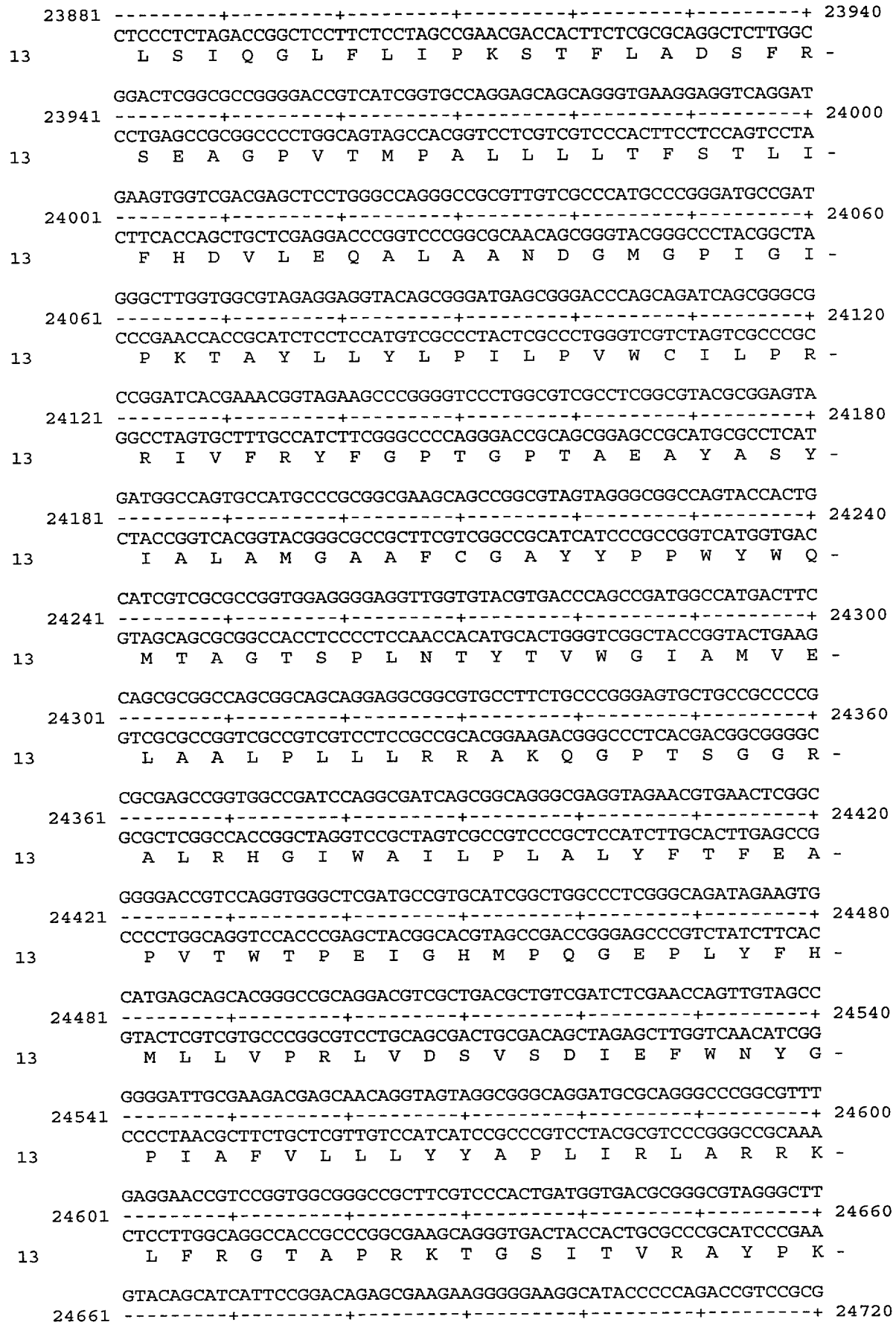

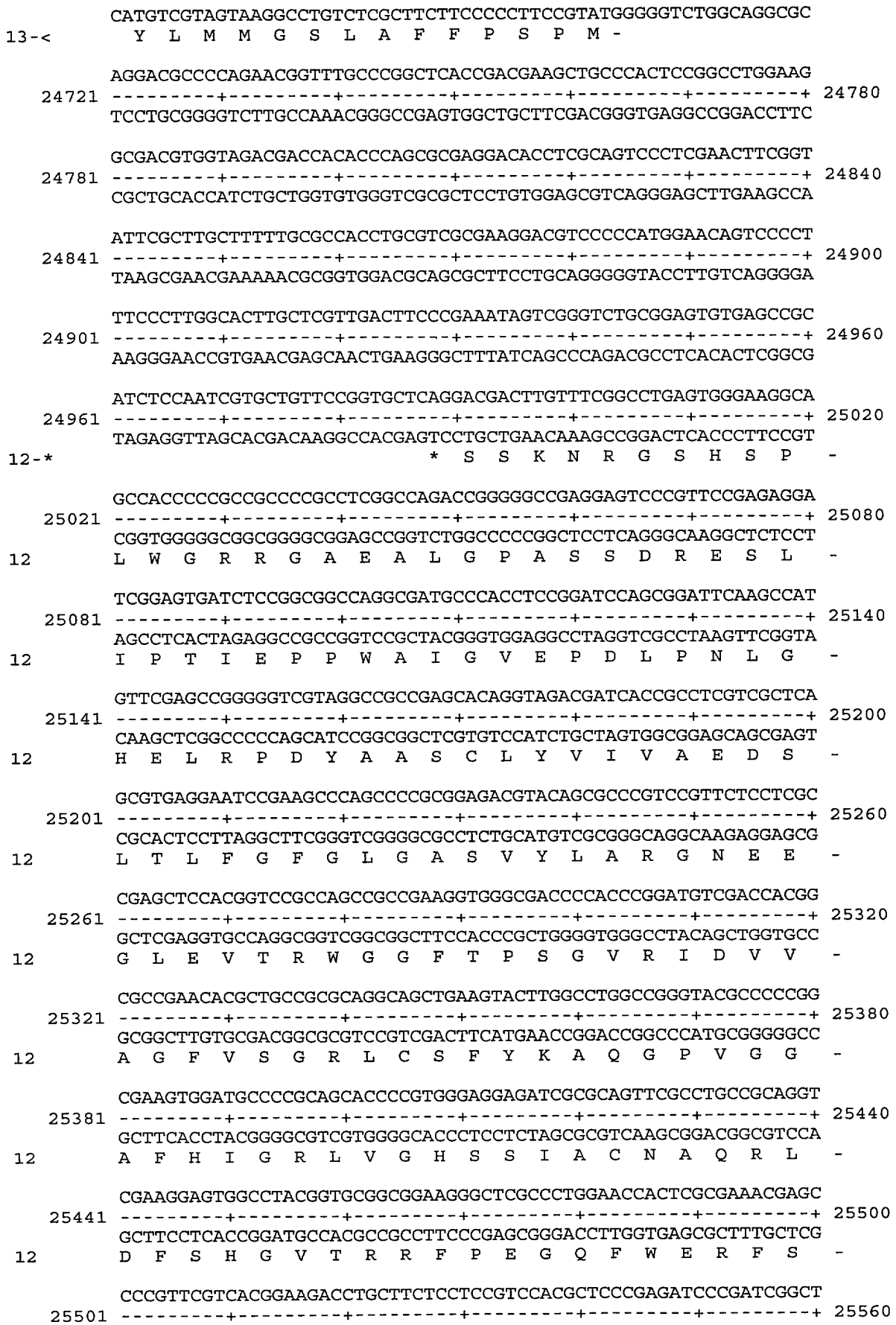

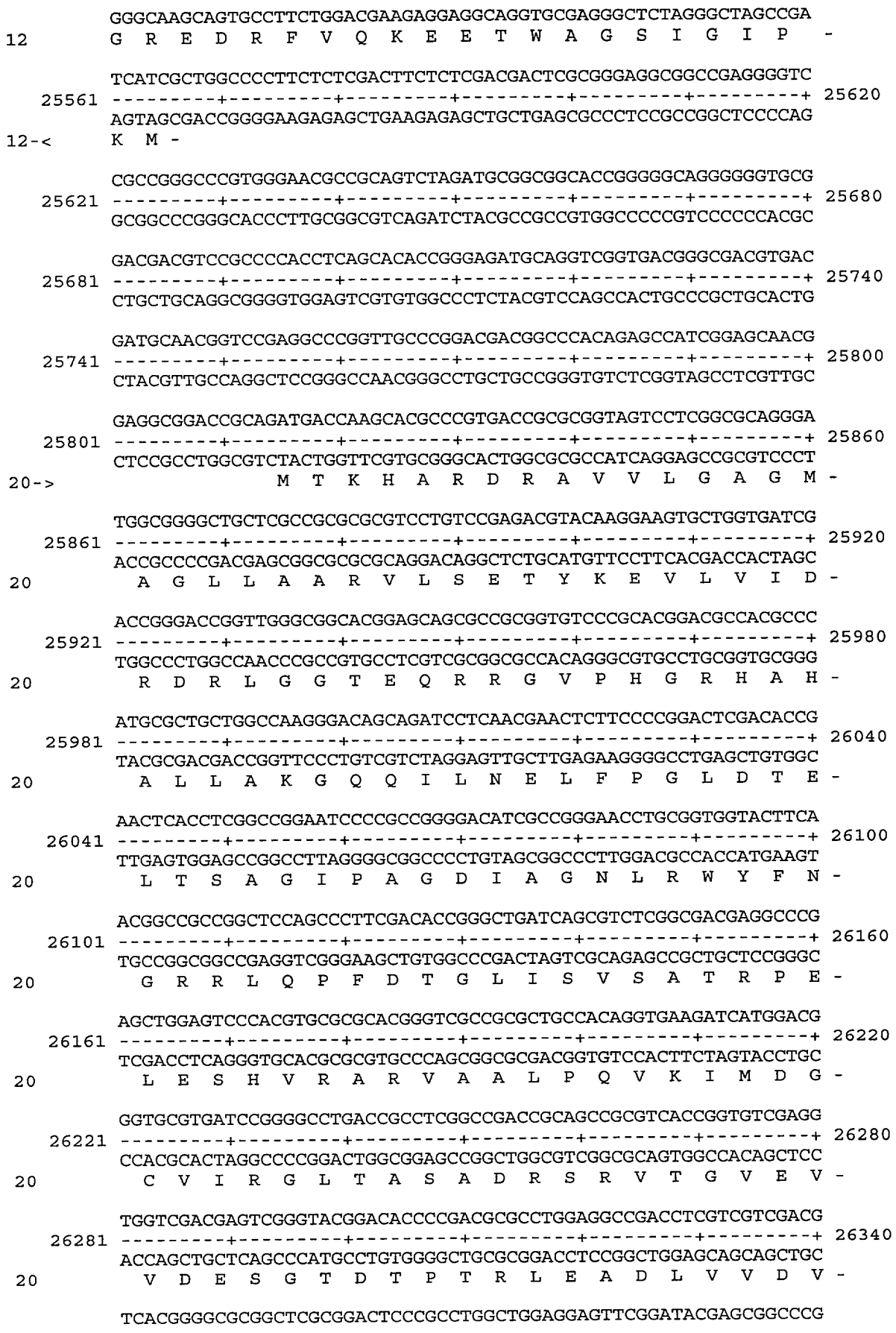

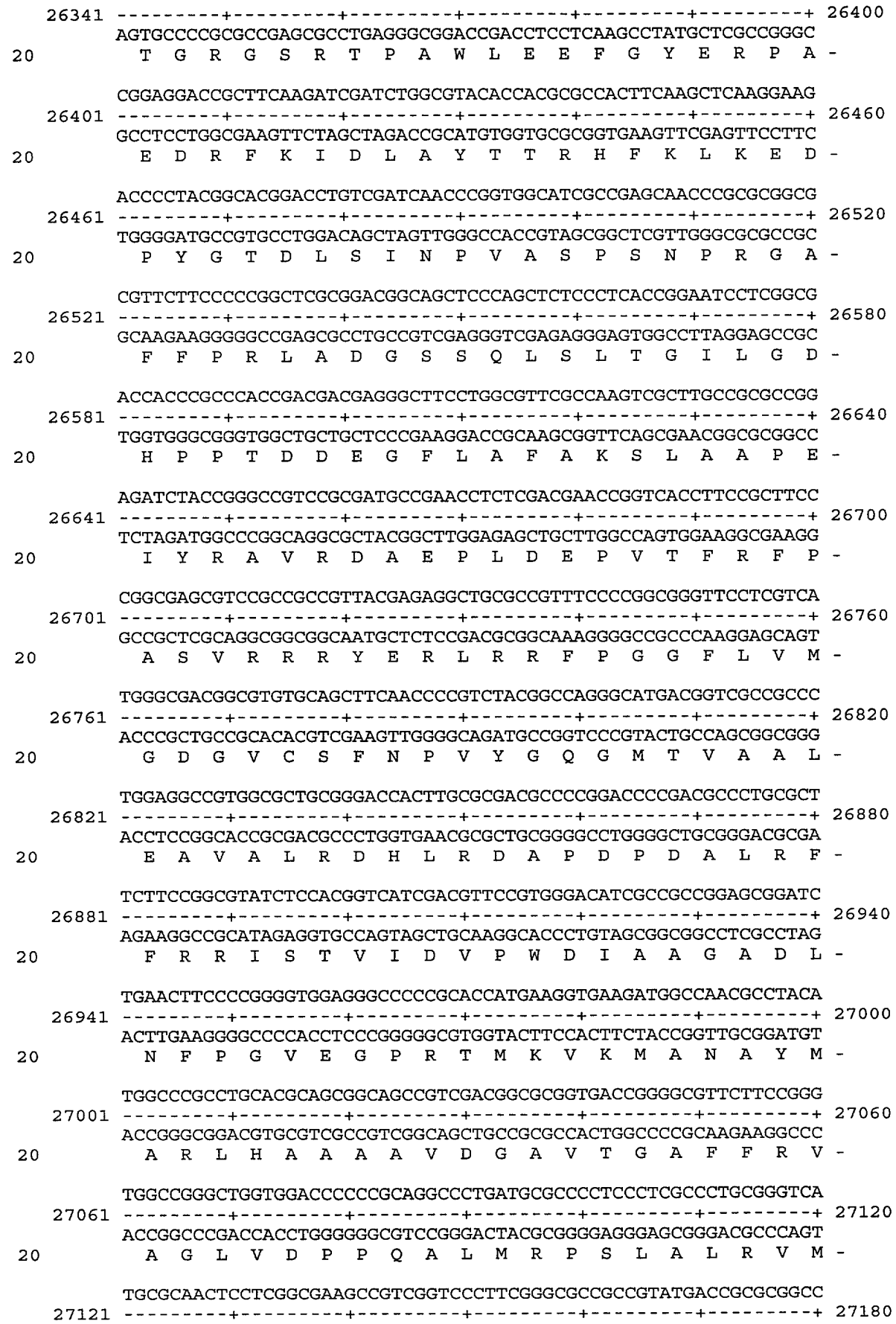

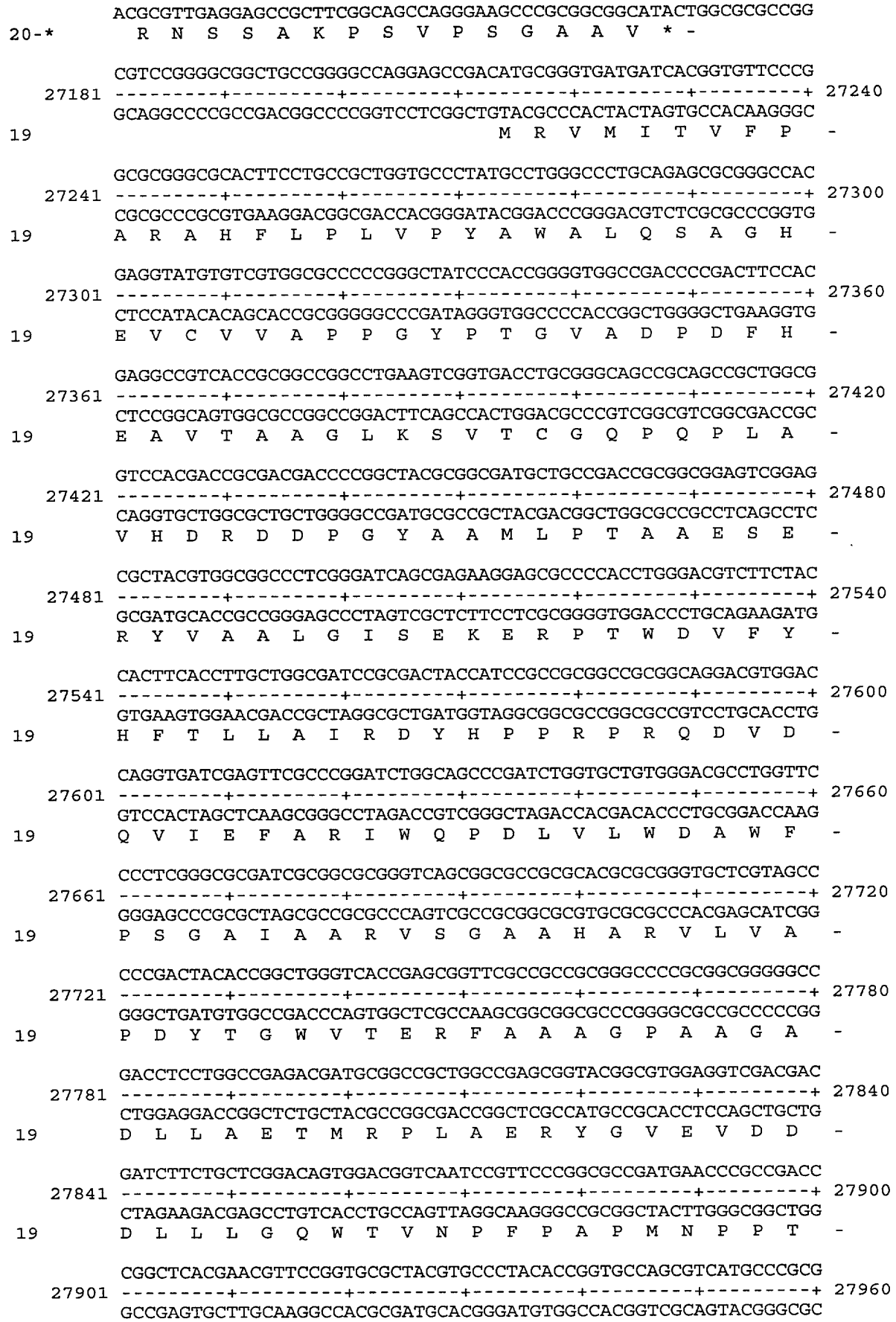
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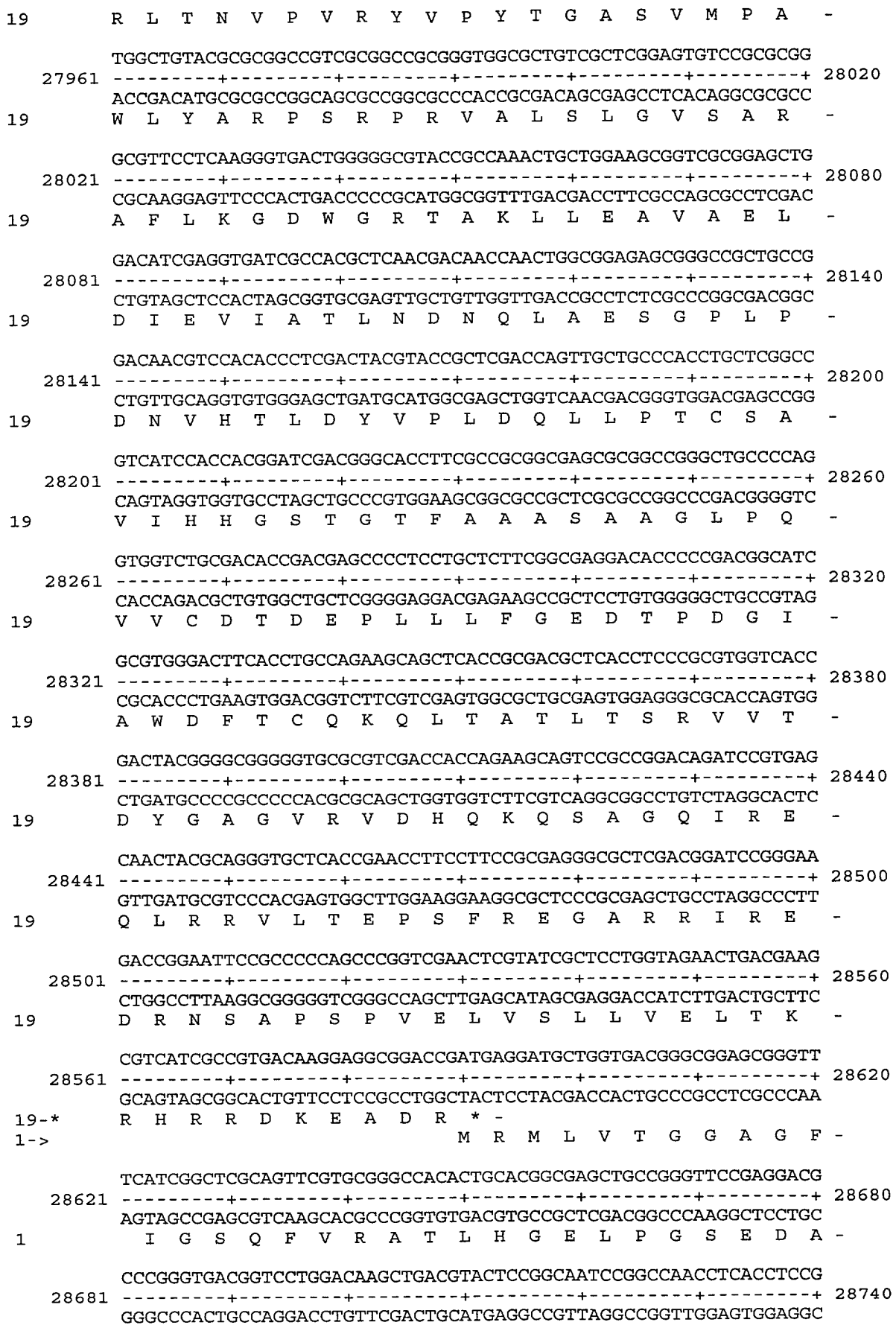
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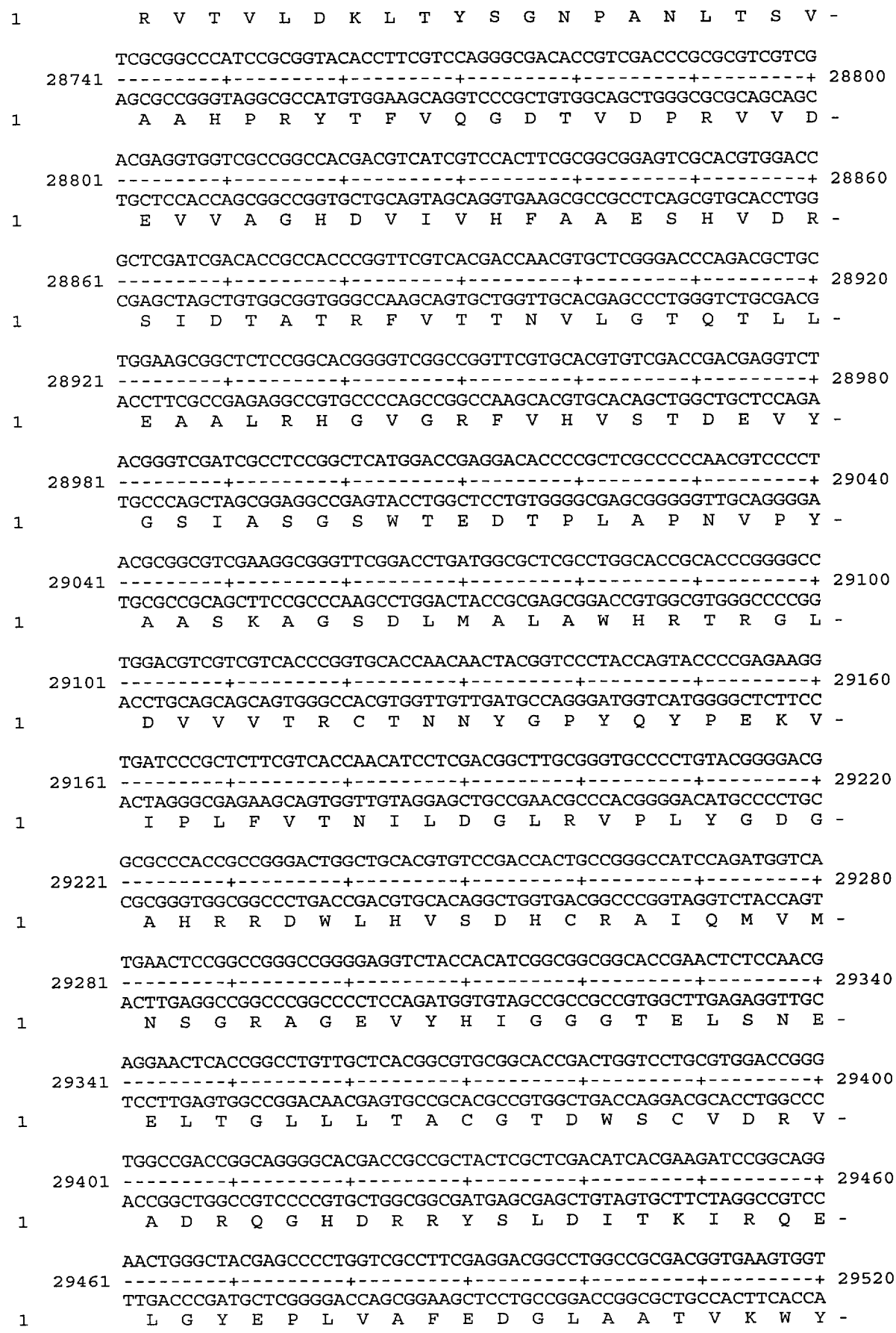

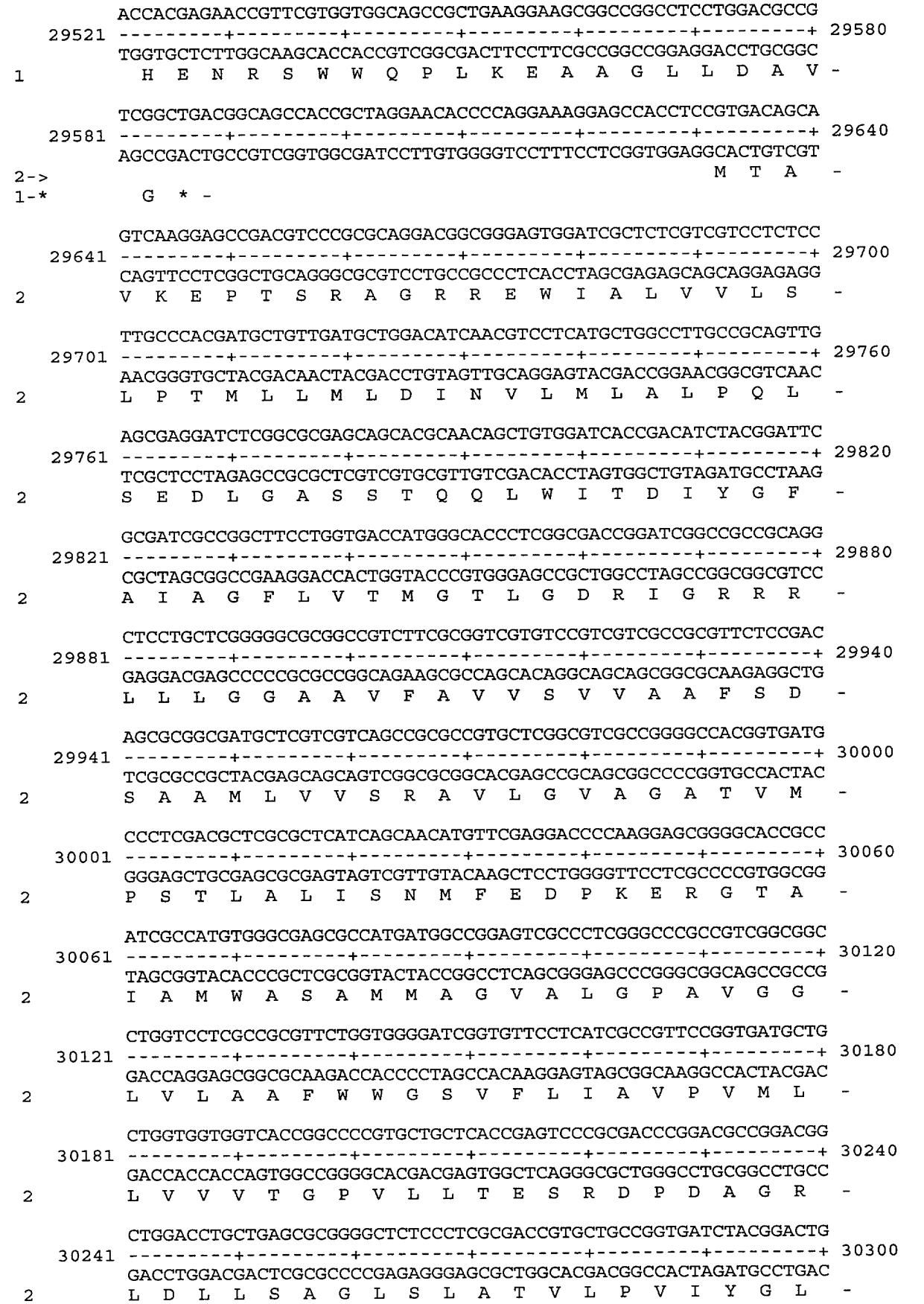

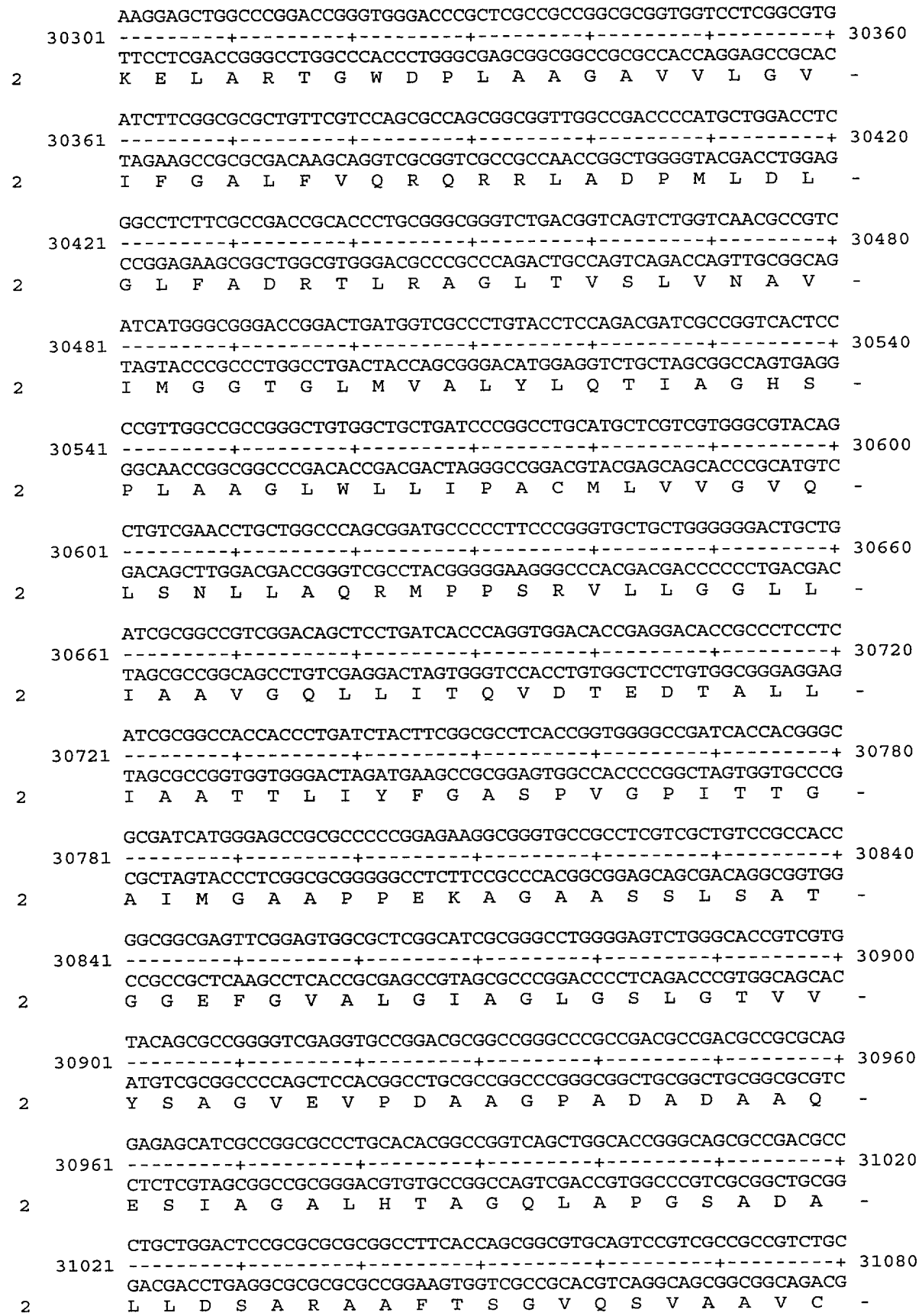

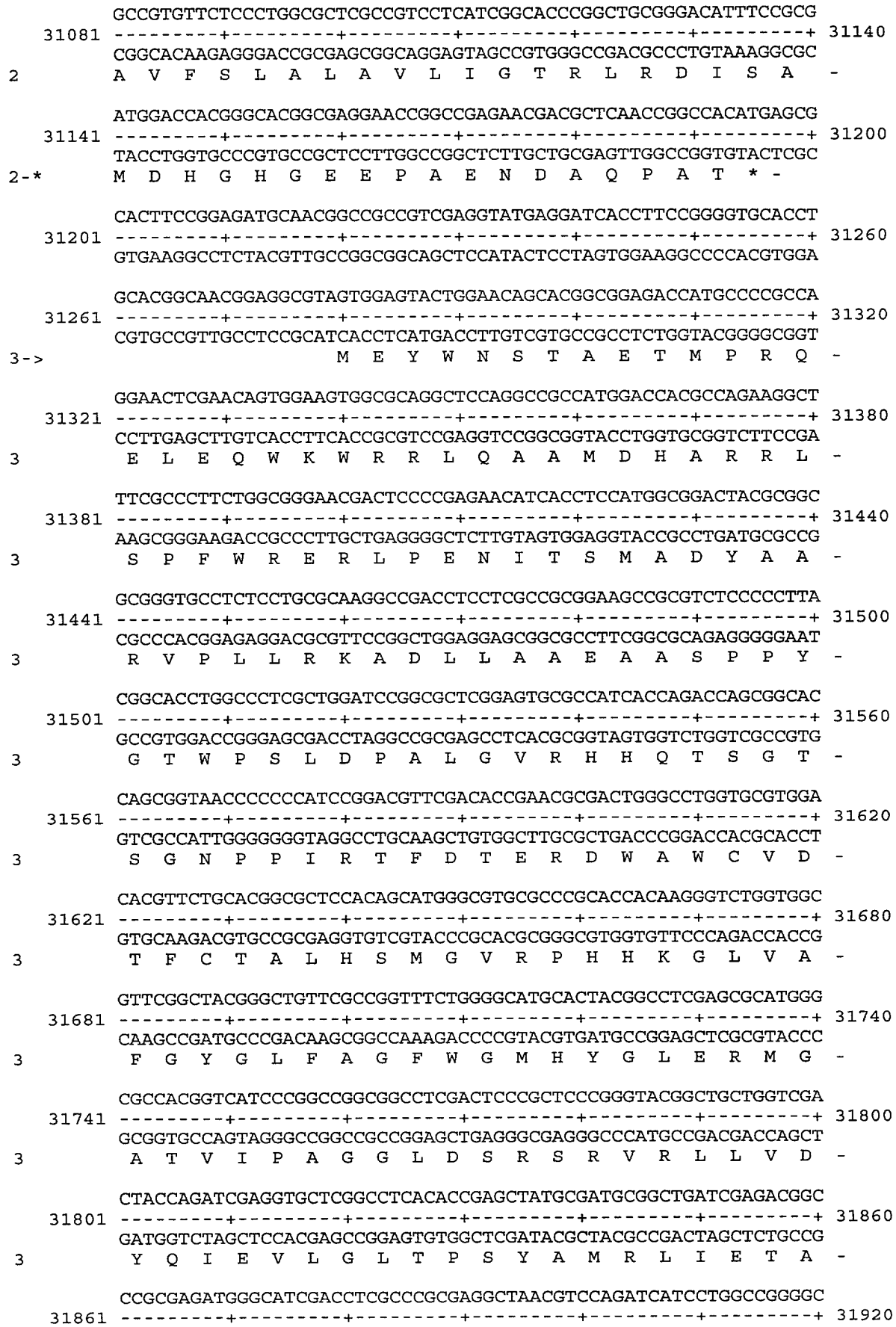
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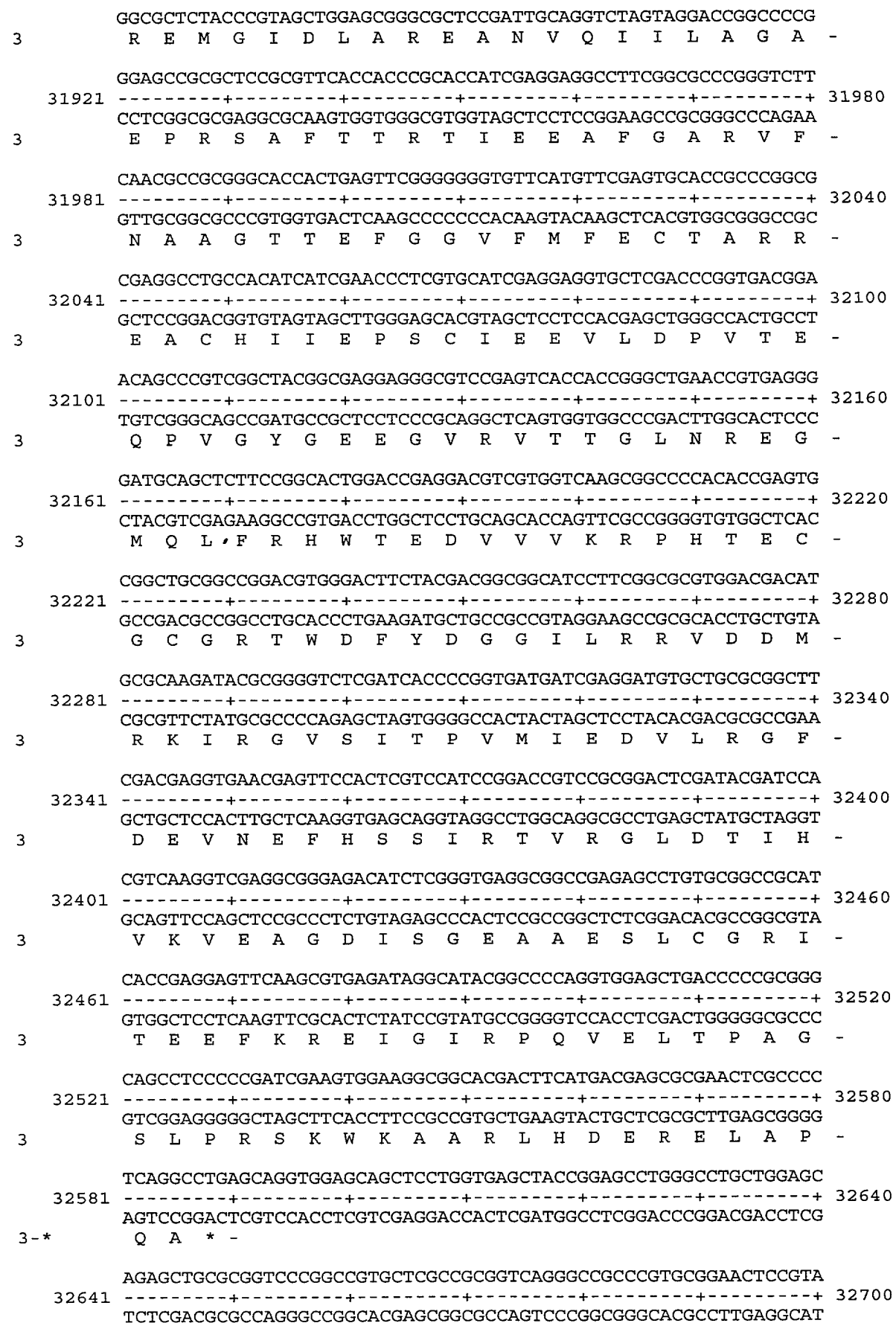

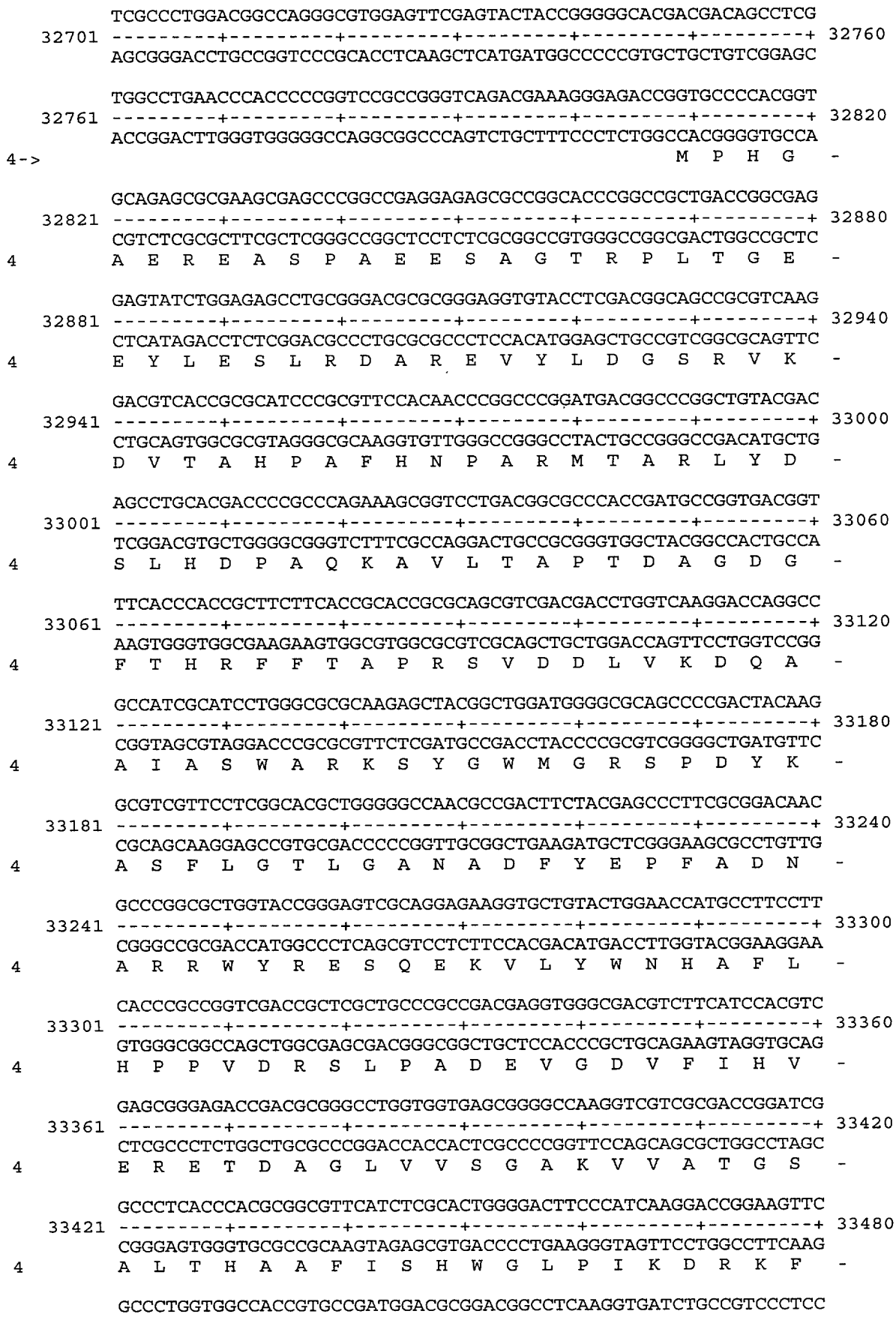

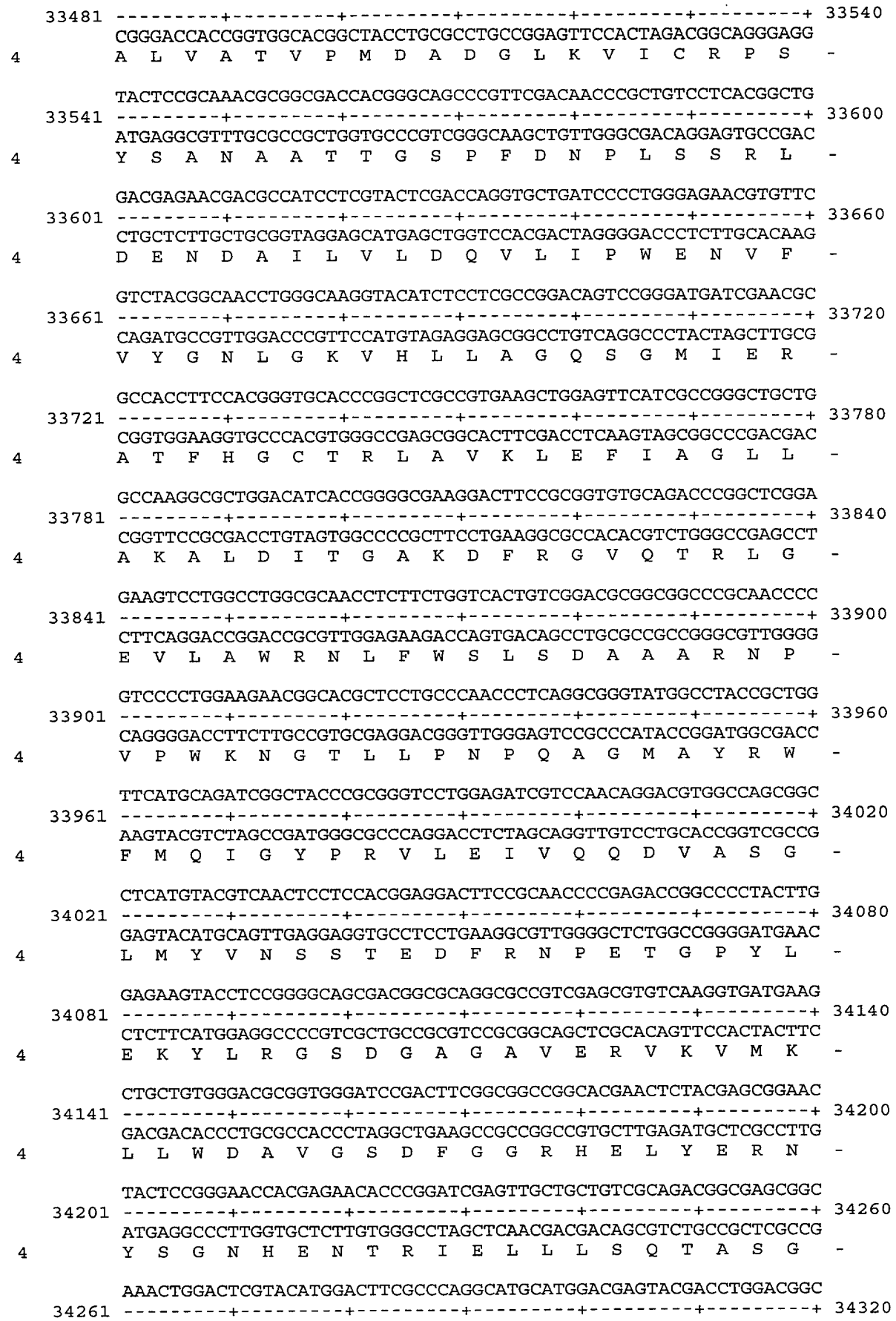
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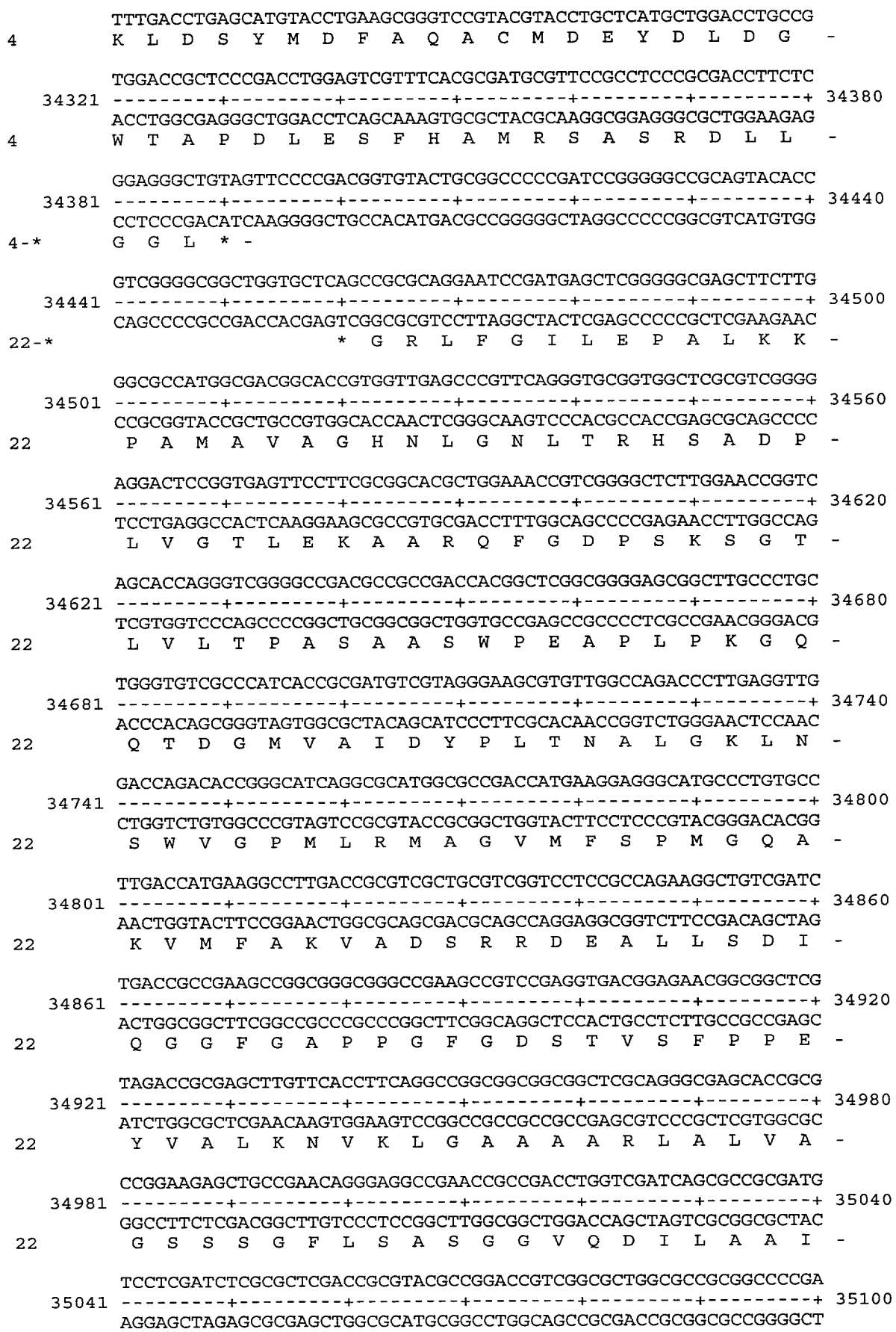
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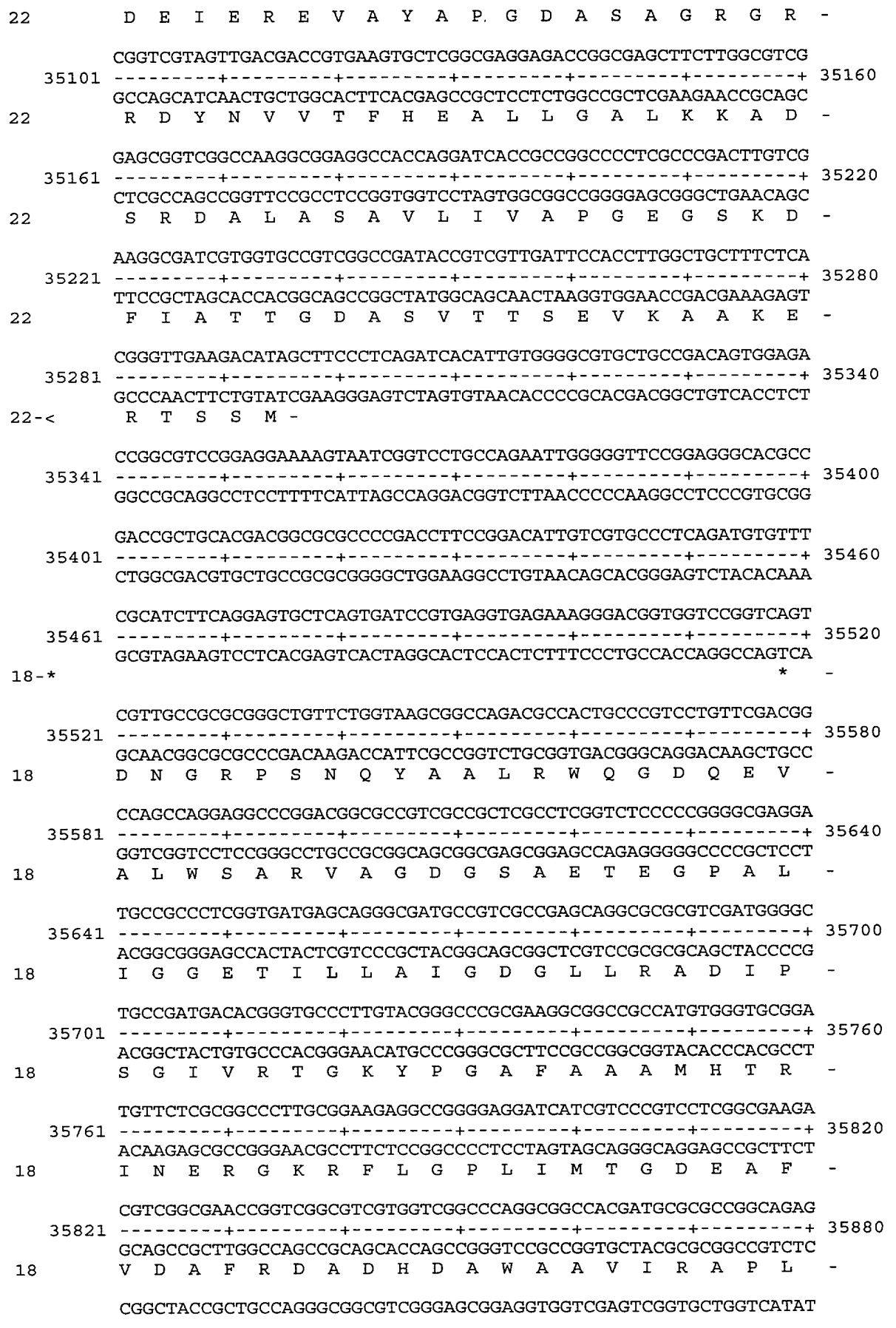
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35161 ---------+---------+---------+---------+---------+---------+ 35220
CTCGCCAGCCGGTTCCGCCTCCGGTGGTCCTAGTGGCGGCCGGGGAGCGGGCTGAACAGC
22 S R D A L A S A V L I V A P G E G S K D -
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35221 ---------+---------+---------+---------+---------+---------+ 35280
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22 F I A T T G D A S V T T S E V K A A K E -
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35281 ---------+---------+---------+---------+---------+---------+ 35340
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22-< R T S S M -
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35341 ---------+---------+---------+---------+---------+---------+ 35400
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35401 ---------+---------+---------+---------+---------+---------+ 35460
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35461 ---------+---------+---------+---------+---------+---------+ 35520
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18-* *
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35521 ---------+---------+---------+---------+---------+---------+ 35580
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18 D N G R P S N Q Y A A L R W Q G D Q E V -
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35581 ---------+---------+---------+---------+---------+---------+ 35640
GGTCGGTCCTCCGGGCCTGCCGCGGCAGCGGCGAGCGGAGCCAGAGGGGGCCCCGCTCCT
18 A L W S A R V A G D G S A E T E G P A L -
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35641 ---------+---------+---------+---------+---------+---------+ 35700
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35701 ---------+---------+---------+---------+---------+---------+ 35760
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35761 ---------+---------+---------+---------+---------+---------+ 35820
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35821 ---------+---------+---------+---------+---------+---------+ 35880
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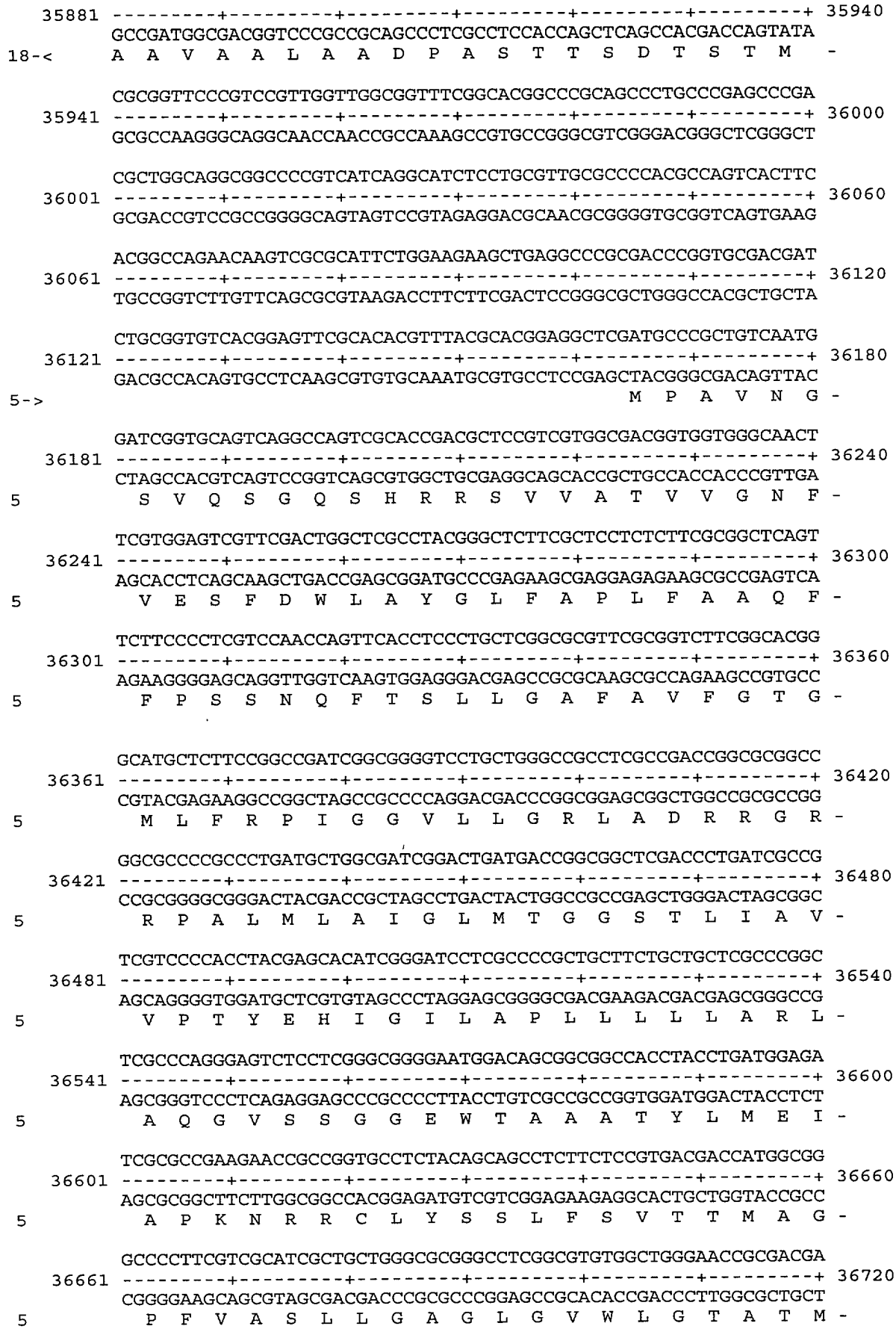

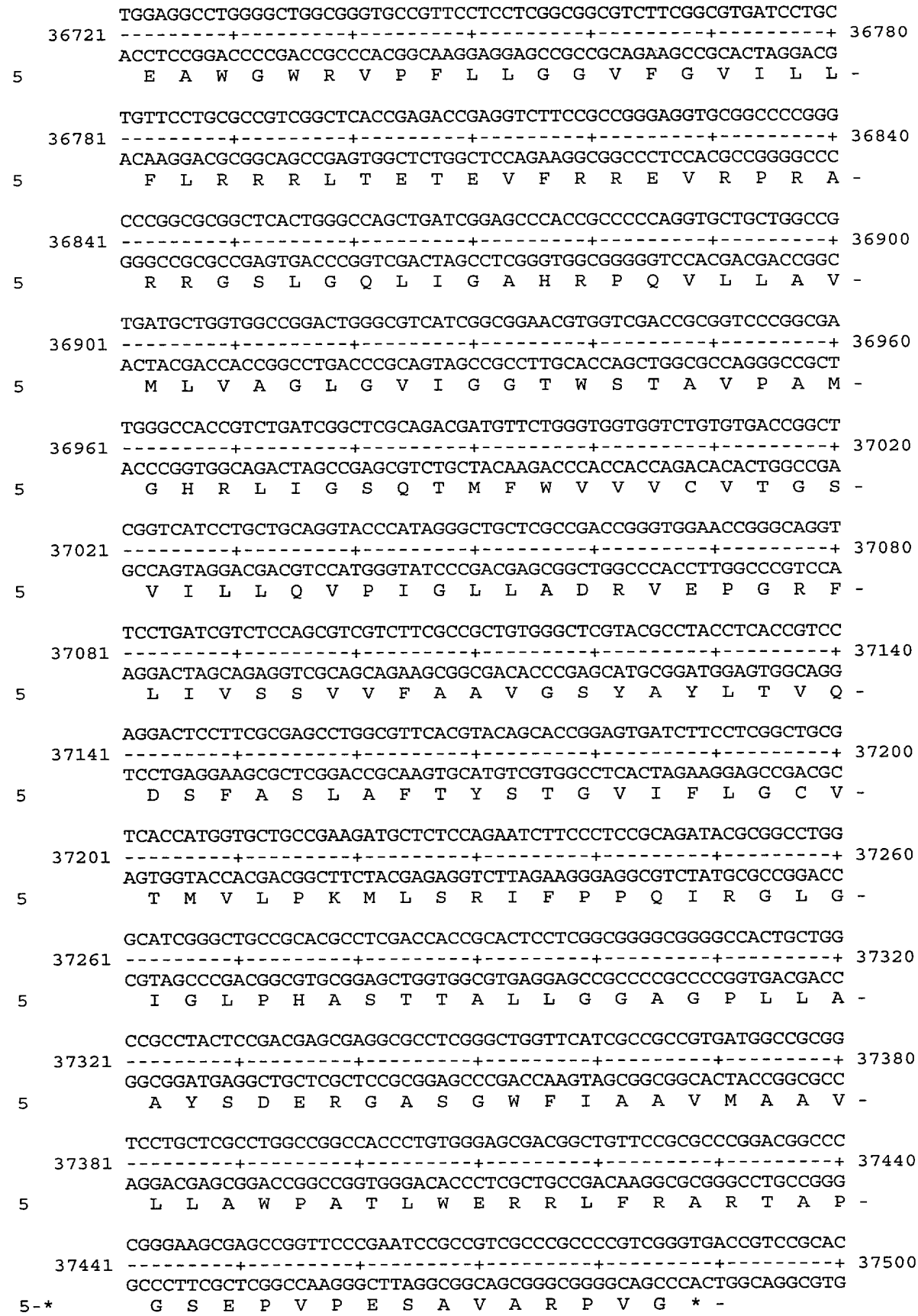

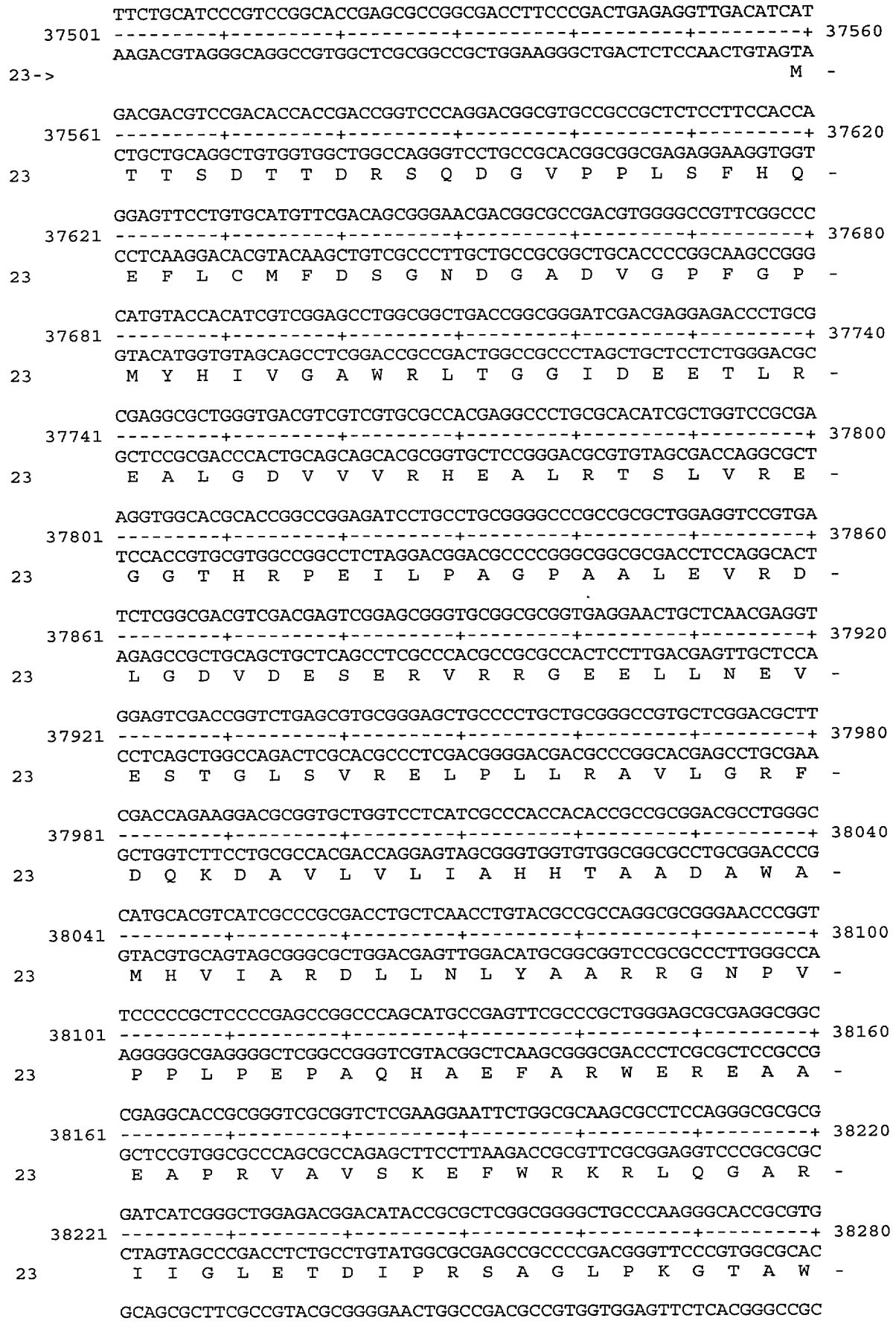
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37741 --------+---------+---------+---------+---------+---------+ 37800
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38041 --------+---------+---------+---------+---------+---------+ 38100
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38101 --------+---------+---------+---------+---------+---------+ 38160
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23 P P L P E P A Q H A E F A R W E R E A A -
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38161 --------+---------+---------+---------+---------+---------+ 38220
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38221 --------+---------+---------+---------+---------+---------+ 38280
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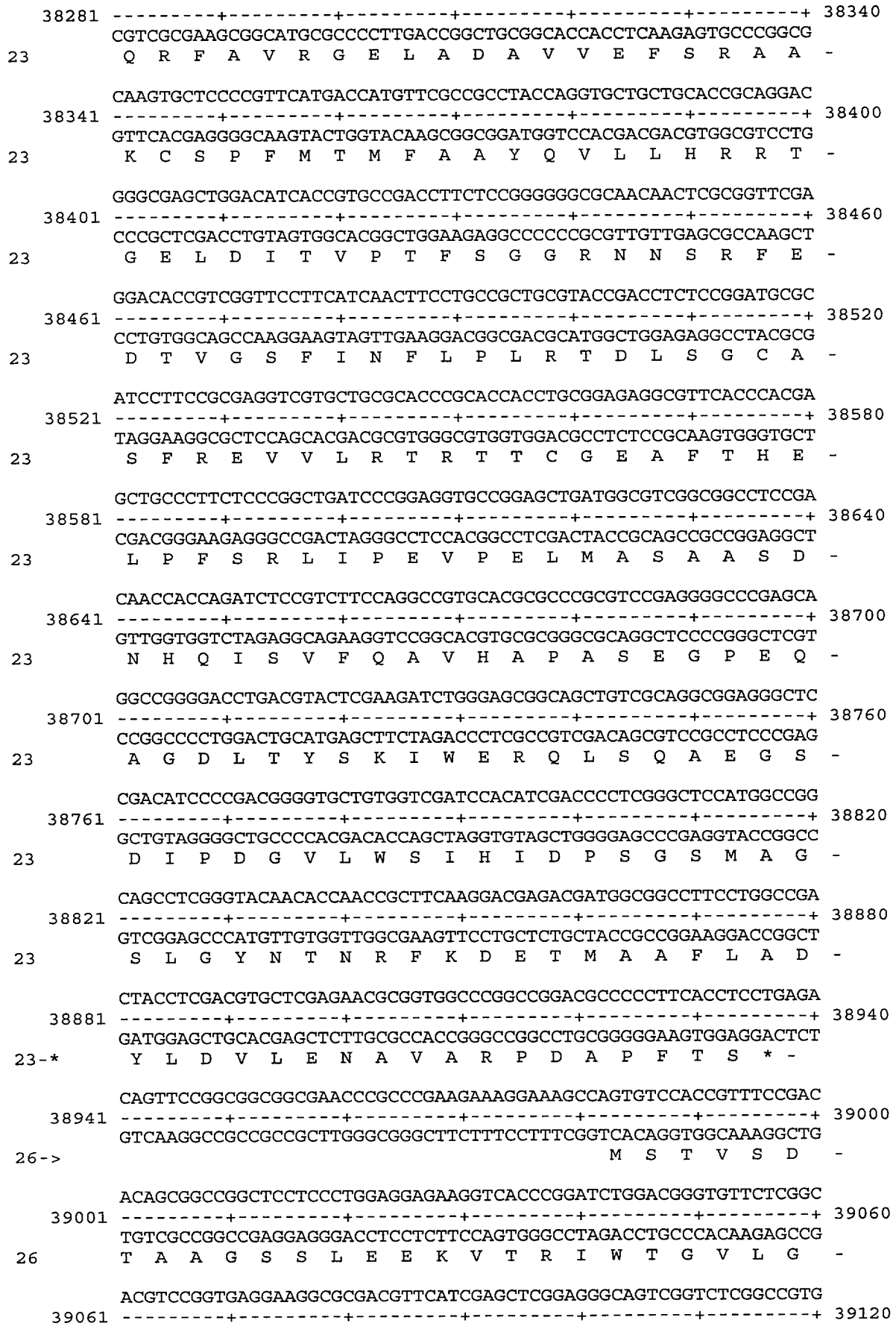
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38341 38400
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23 K C S P F M T M F A A Y Q V L L H R R T -
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38401 38460
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38461 38520
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38521 38580
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38581 38640
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38641 38700
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23 N H Q I S V F Q A V H A P A S E G P E Q -
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38761 38820
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38821 38880
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23 S L G Y N T N R F K D E T M A A F L A D -
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38881 38940
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38941 39000
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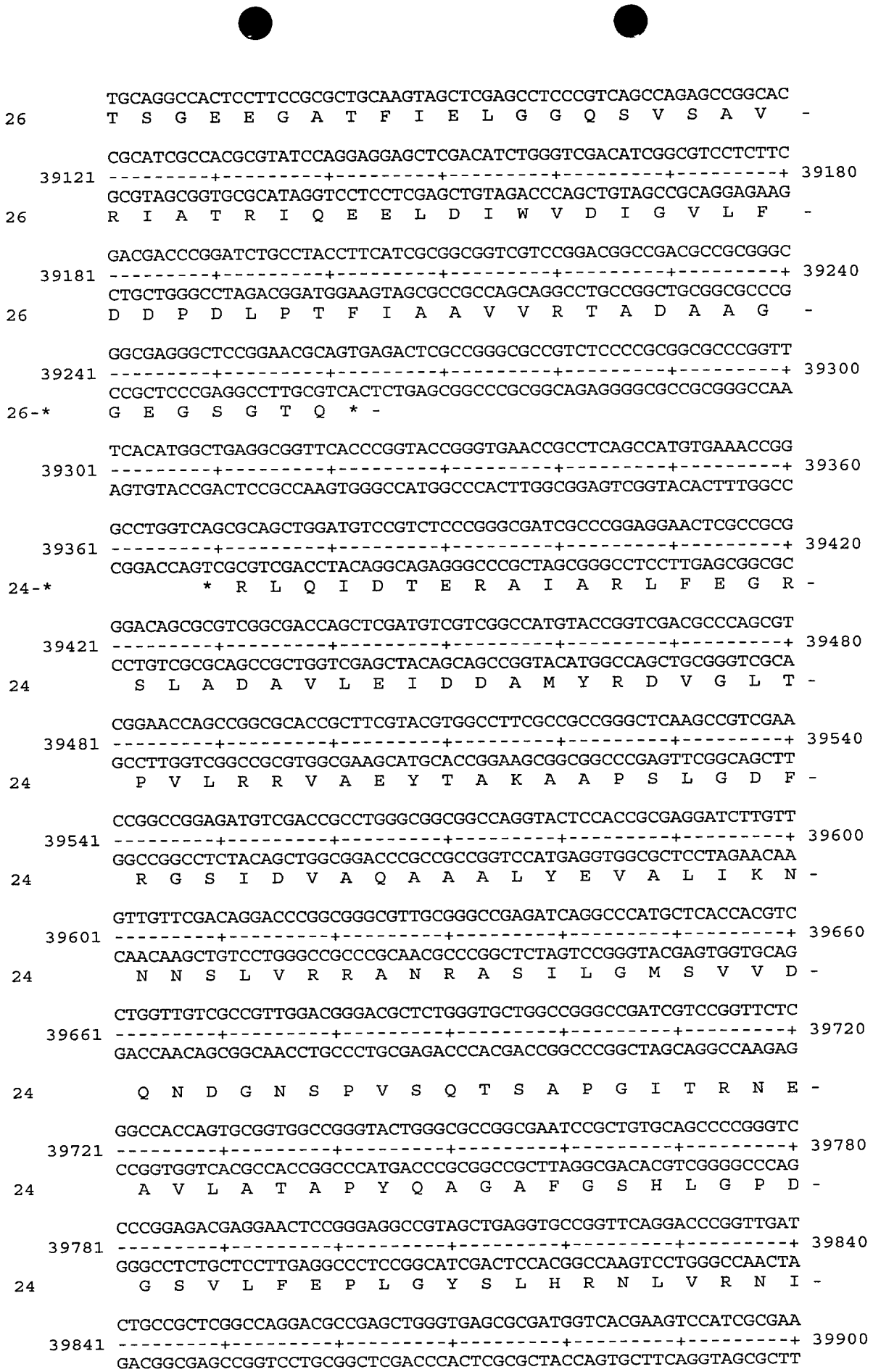

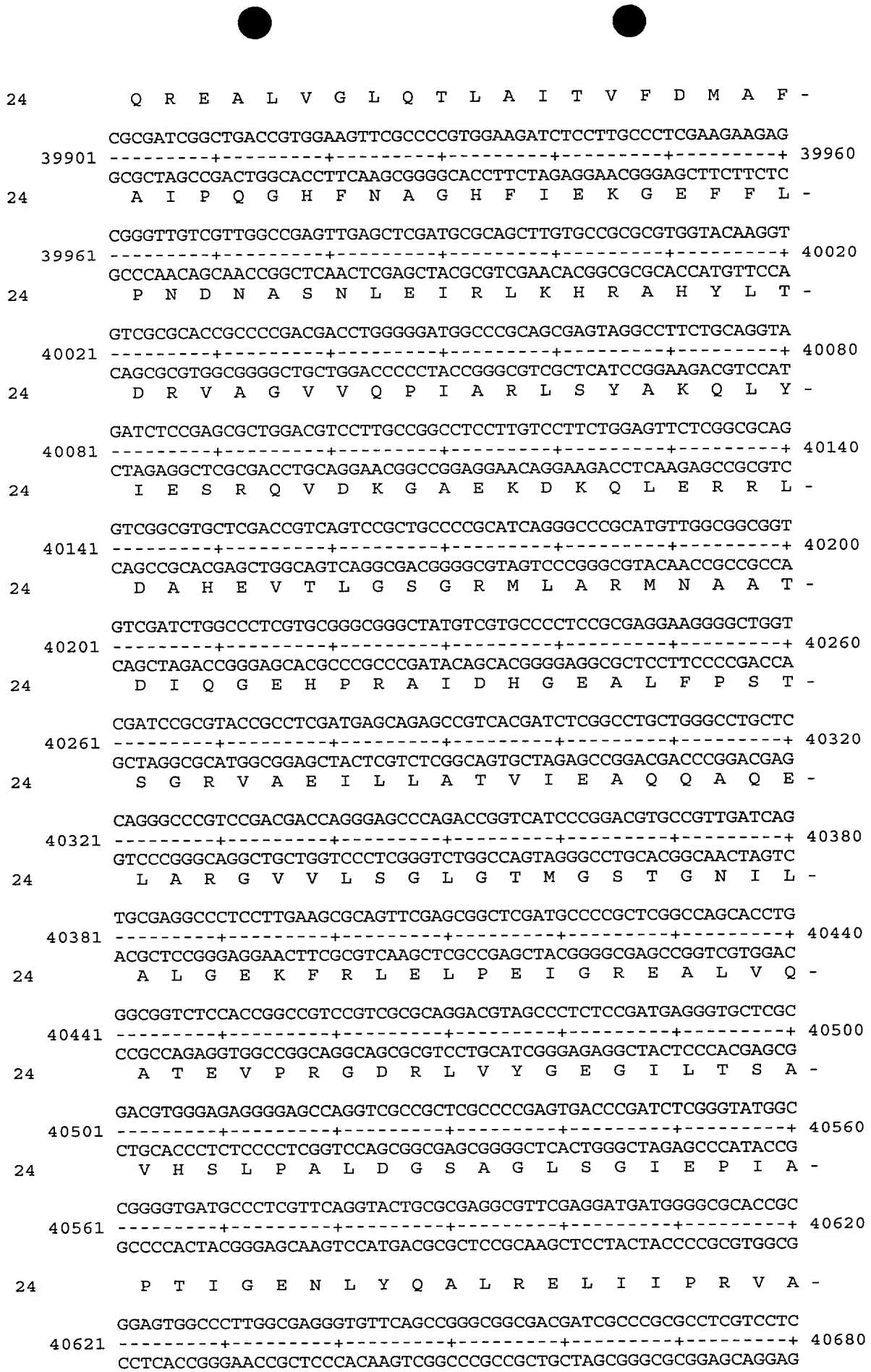
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24 A I P Q G H F N A G H F I E K G E F F L -
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39961 + 40020
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24 P N D N A S N L E I R L K H R A H Y L T -
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40021 + 40080
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24 D R V A G V V Q P I A R L S Y A K Q L Y -
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40081 + 40140
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24 I E S R Q V D K G A E K D K Q L E R R L -
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40141 + 40200
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40321 + 40380
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40501 + 40560
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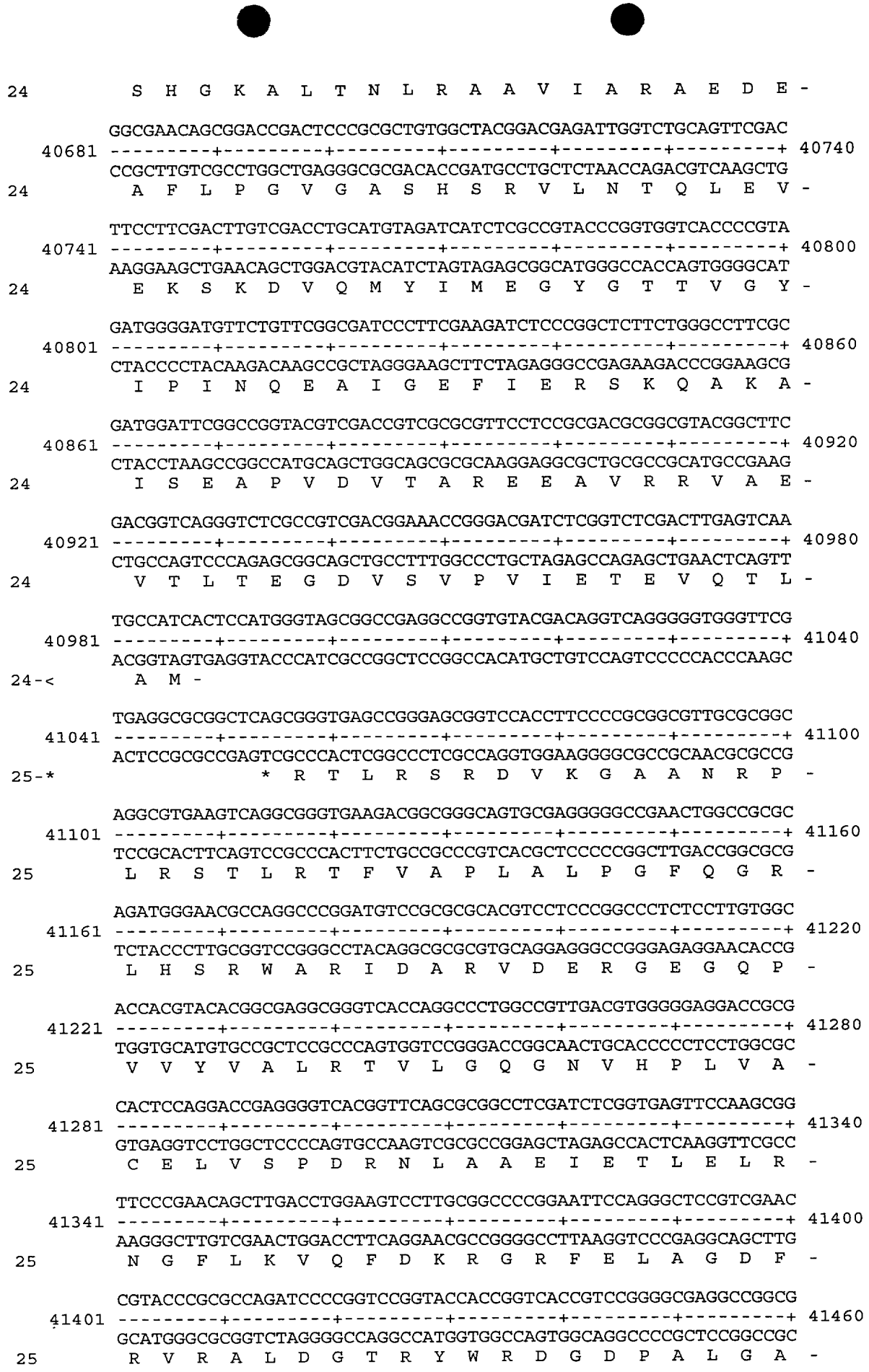

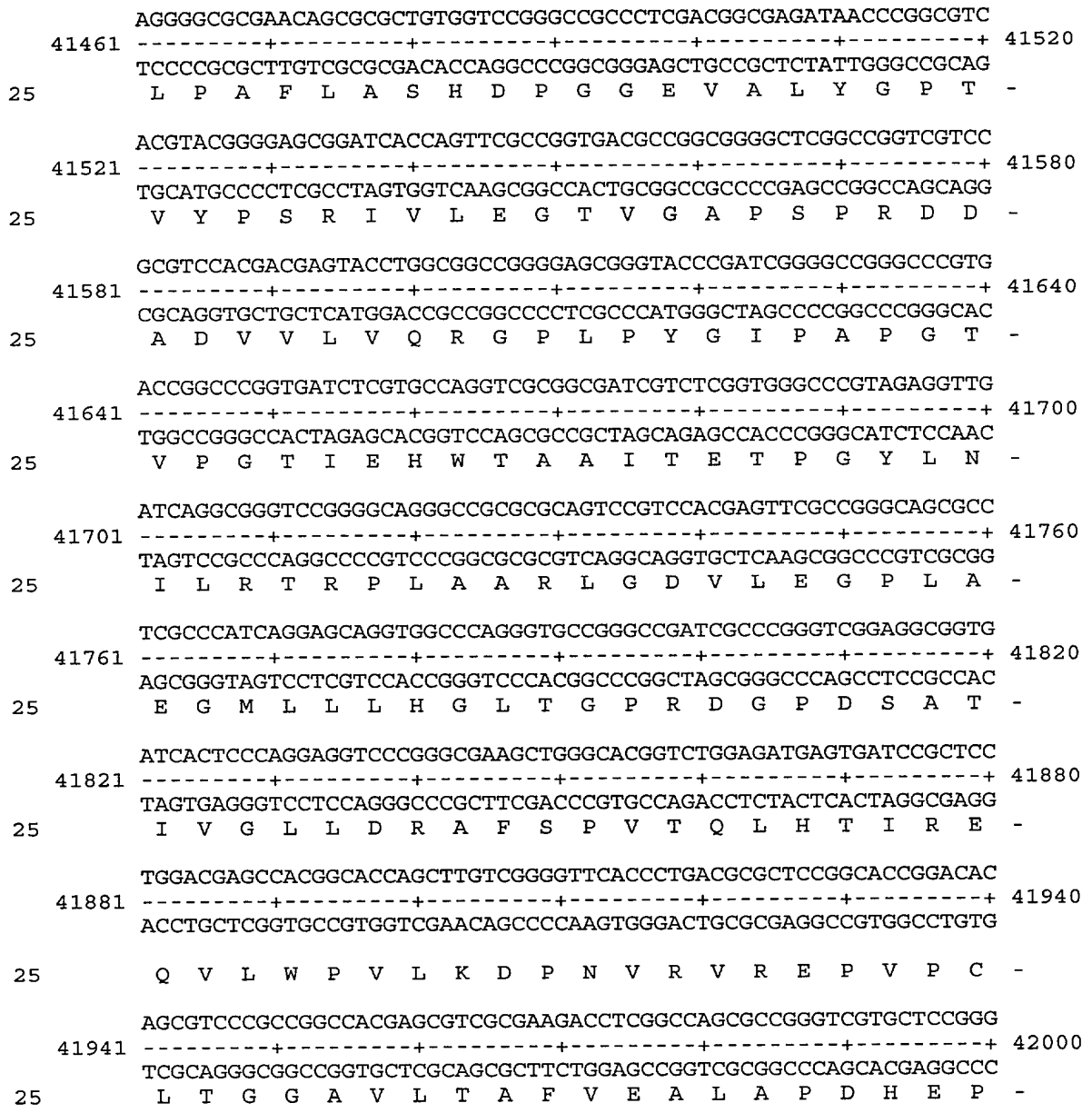

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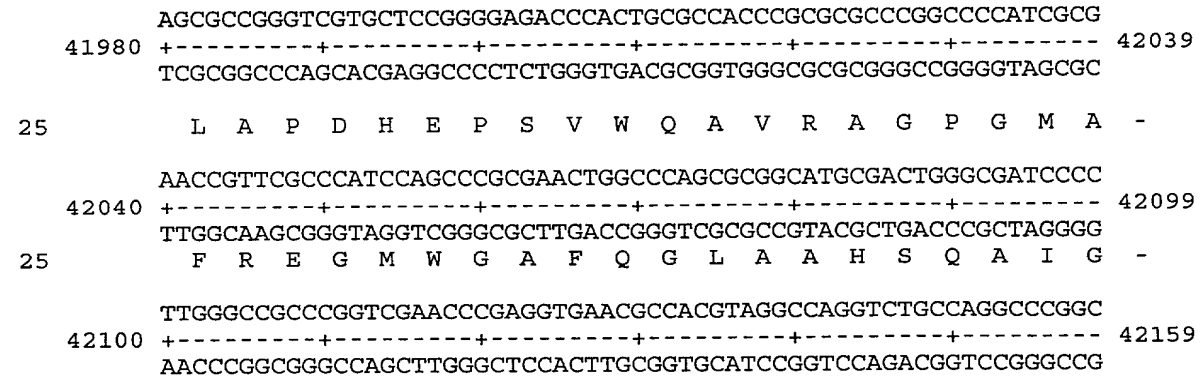

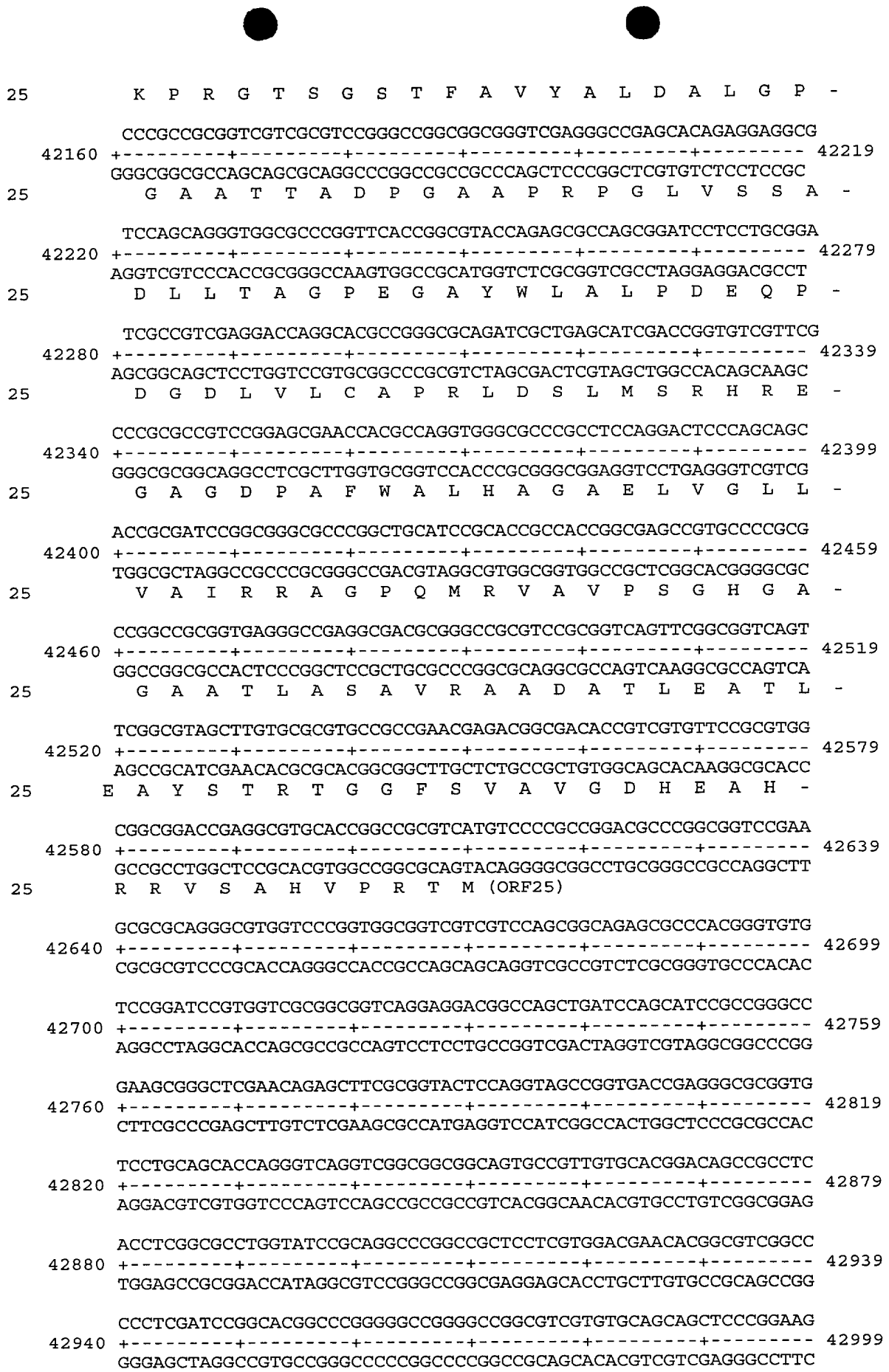

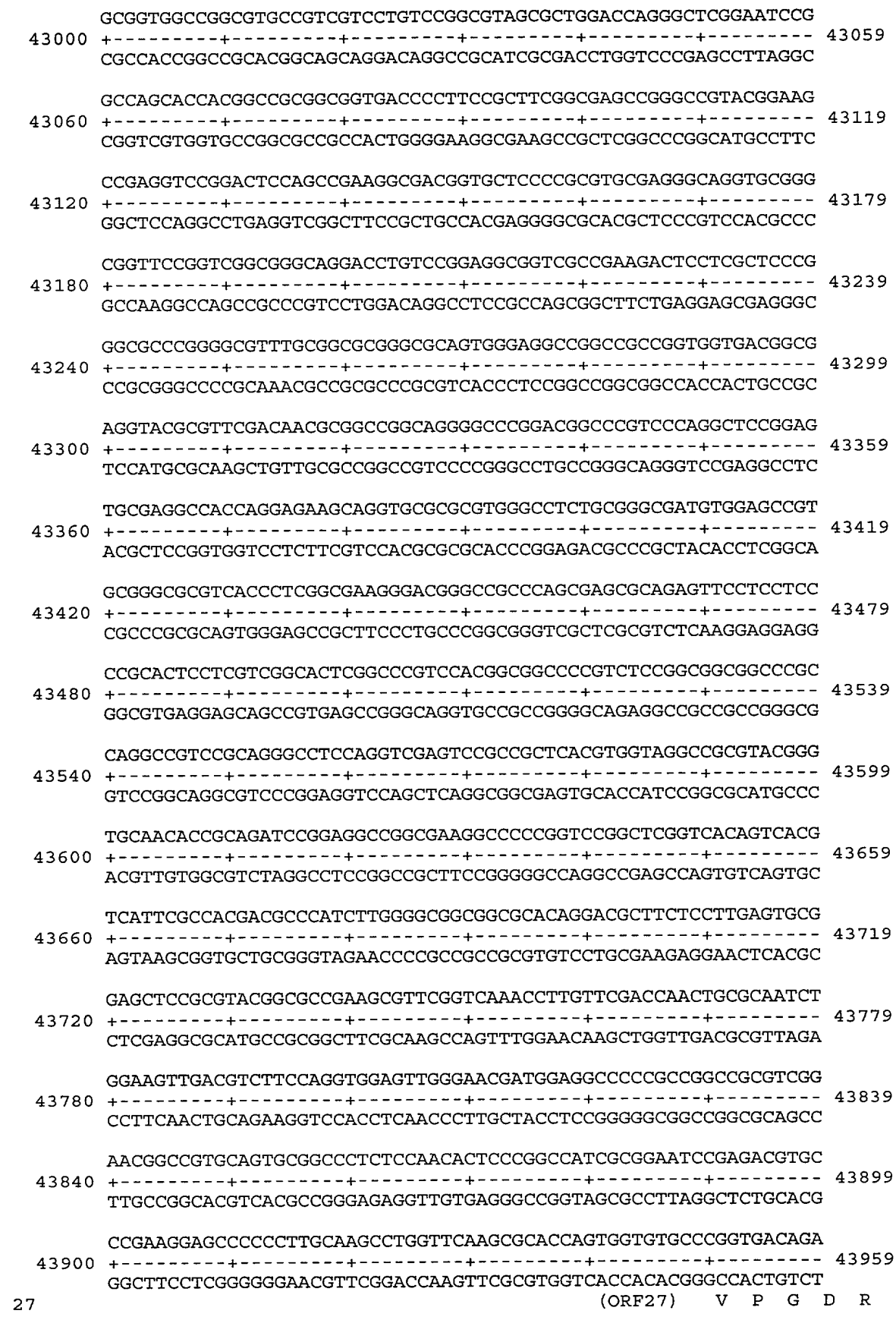
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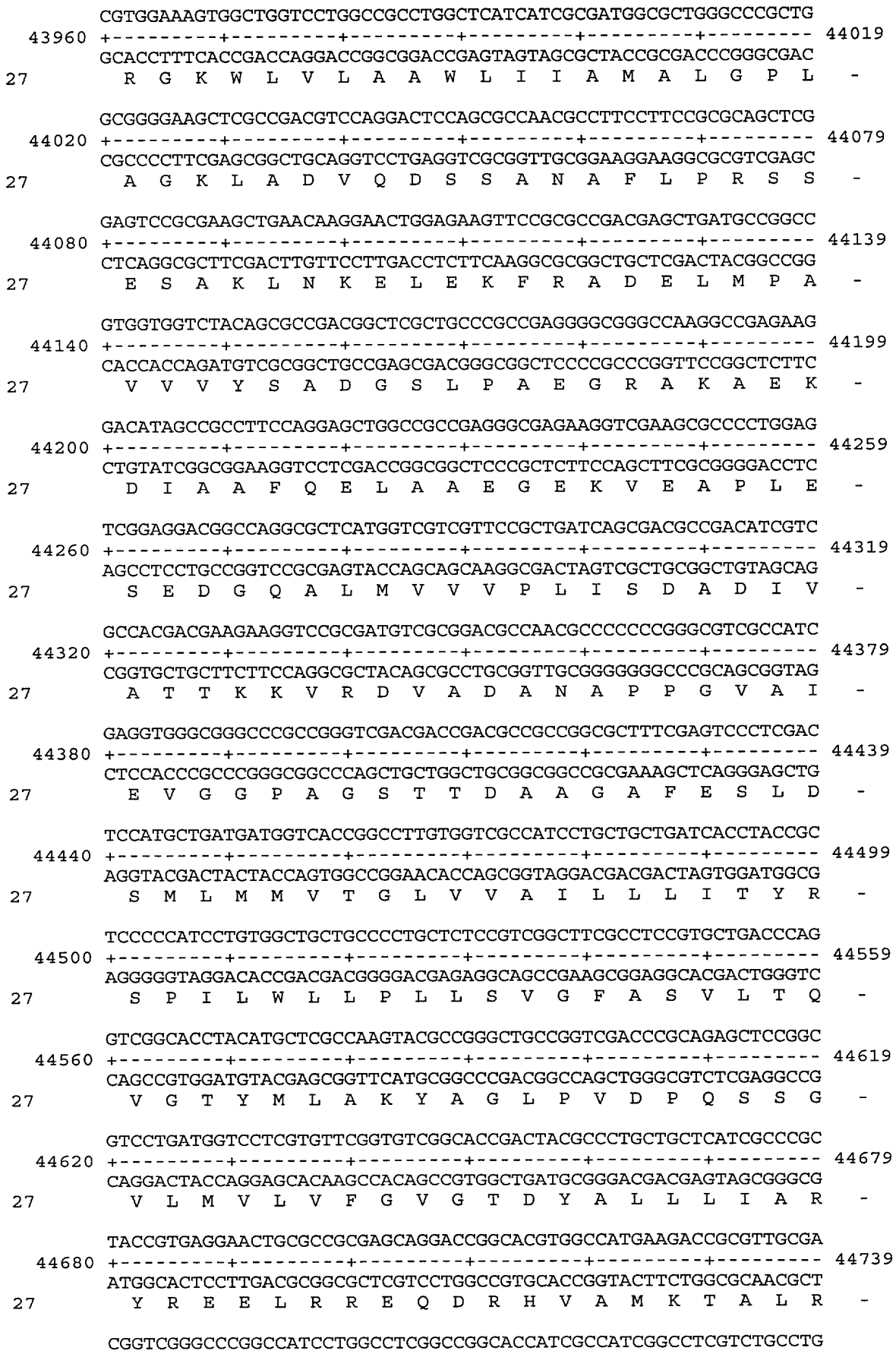
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44020 44079
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44080 44139
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44440 44499
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27 S M L M M V T G L V V A I L L L I T Y R -
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44500 44559
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27 S P I L W L L P L L S V G F A S V L T Q -
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44560 44619
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44620 44679
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27 V L M V L V F G V G T D Y A L L L I A R -
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44680 44739
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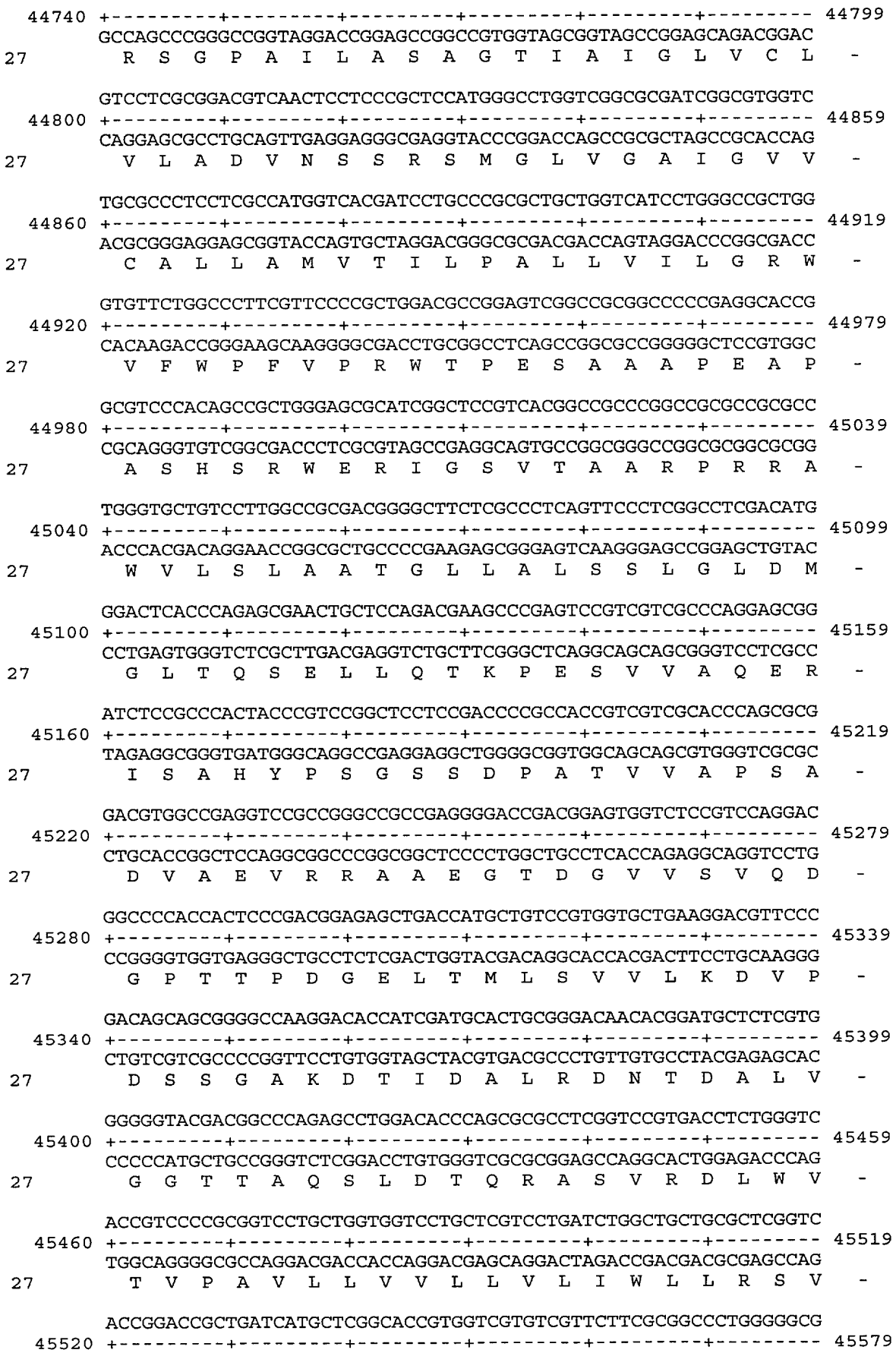

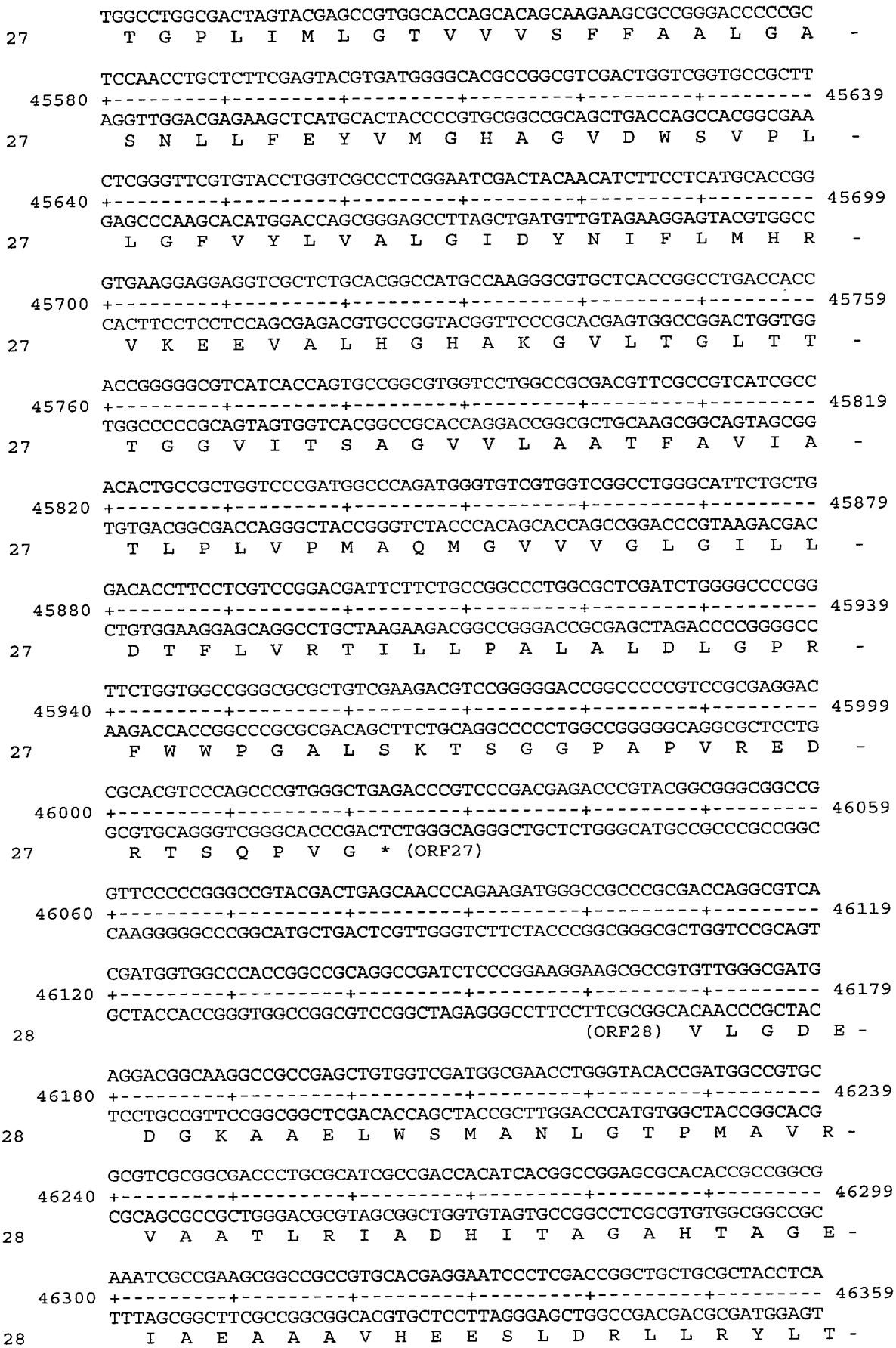

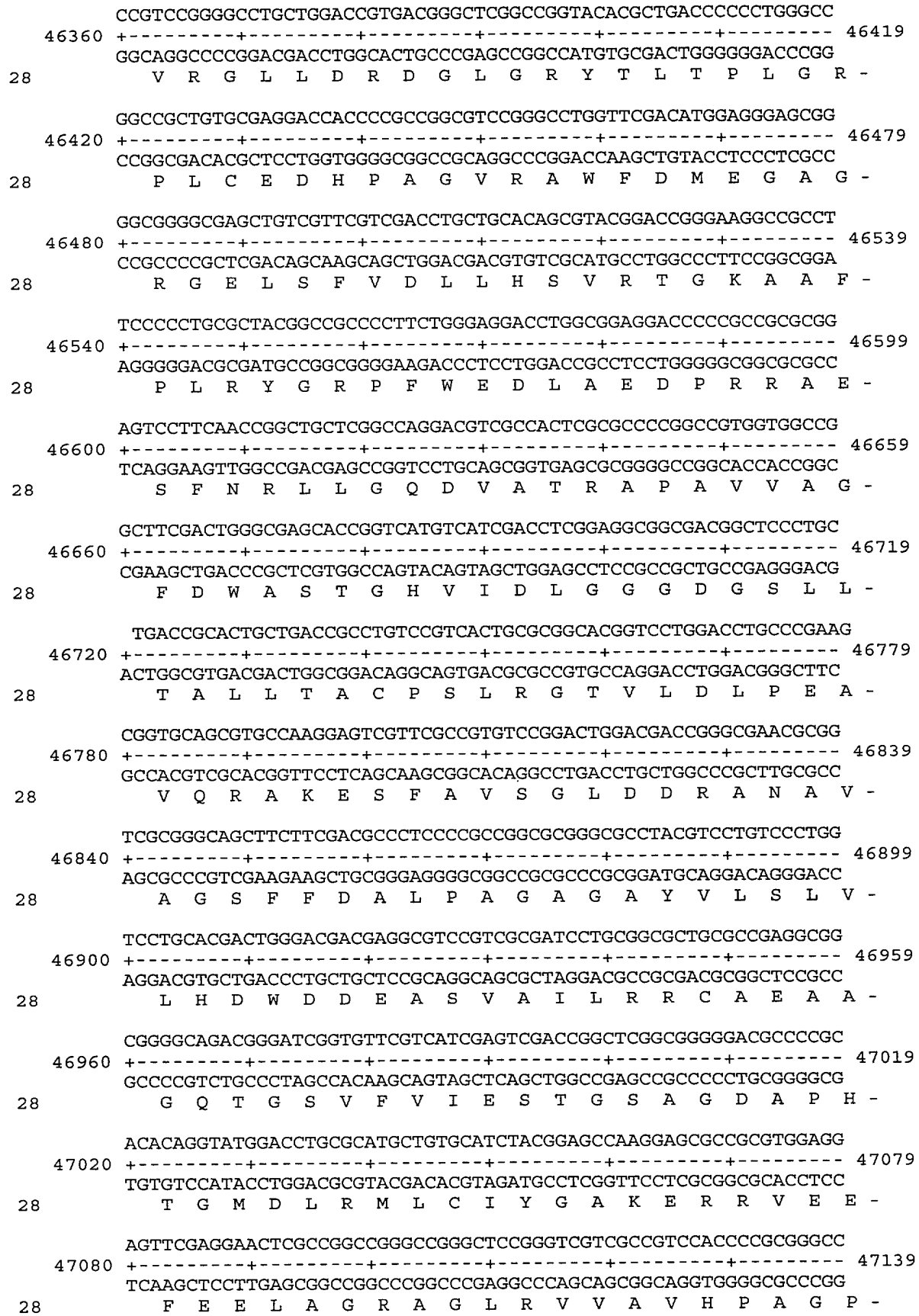
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28 V R G L L D R D G L G R Y T L T P L G R -
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46420 +---------+---------+---------+---------+---------+--------- 46479
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28 P L C E D H P A G V R A W F D M E G A G -
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46480 +---------+---------+---------+---------+---------+--------- 46539
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46540 +---------+---------+---------+---------+---------+--------- 46599
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28 P L R Y G R P F W E D L A E D P R R A E -
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46600 +---------+---------+---------+---------+---------+--------- 46659
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28 S F N R L L G Q D V A T R A P A V V A G -
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28 T A L L T A C P S L R G T V L D L P E A -
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46780 +---------+---------+---------+---------+---------+--------- 46839
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28 V Q R A K E S F A V S G L D D R A N A V -
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46840 +---------+---------+---------+---------+---------+--------- 46899
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28 A G S F F D A L P A G A G A Y V L S L V -
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28 G Q T G S V F V I E S T G S A G D A P H -
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47020 +---------+---------+---------+---------+---------+--------- 47079
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47080 +---------+---------+---------+---------+---------+--------- 47139
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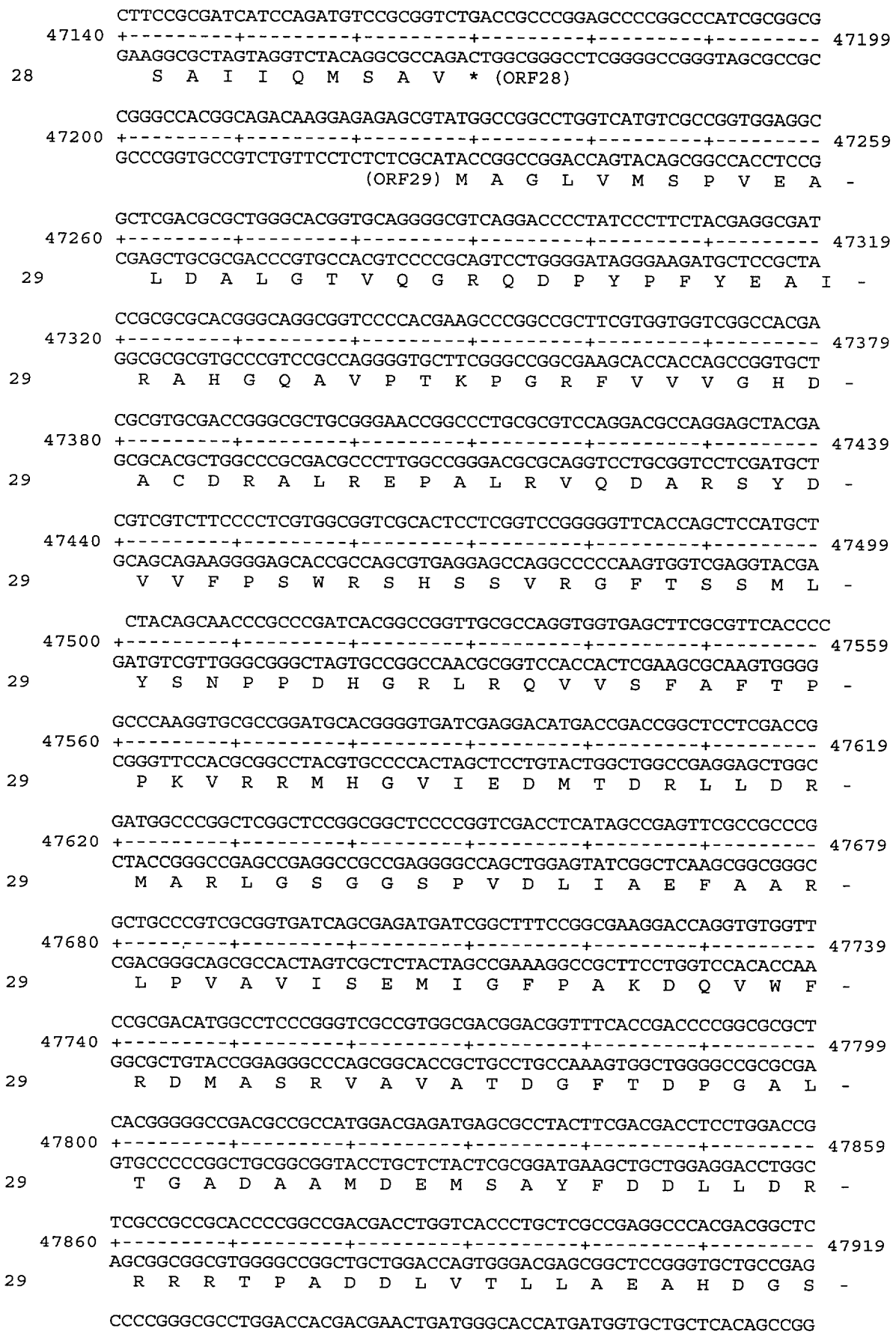

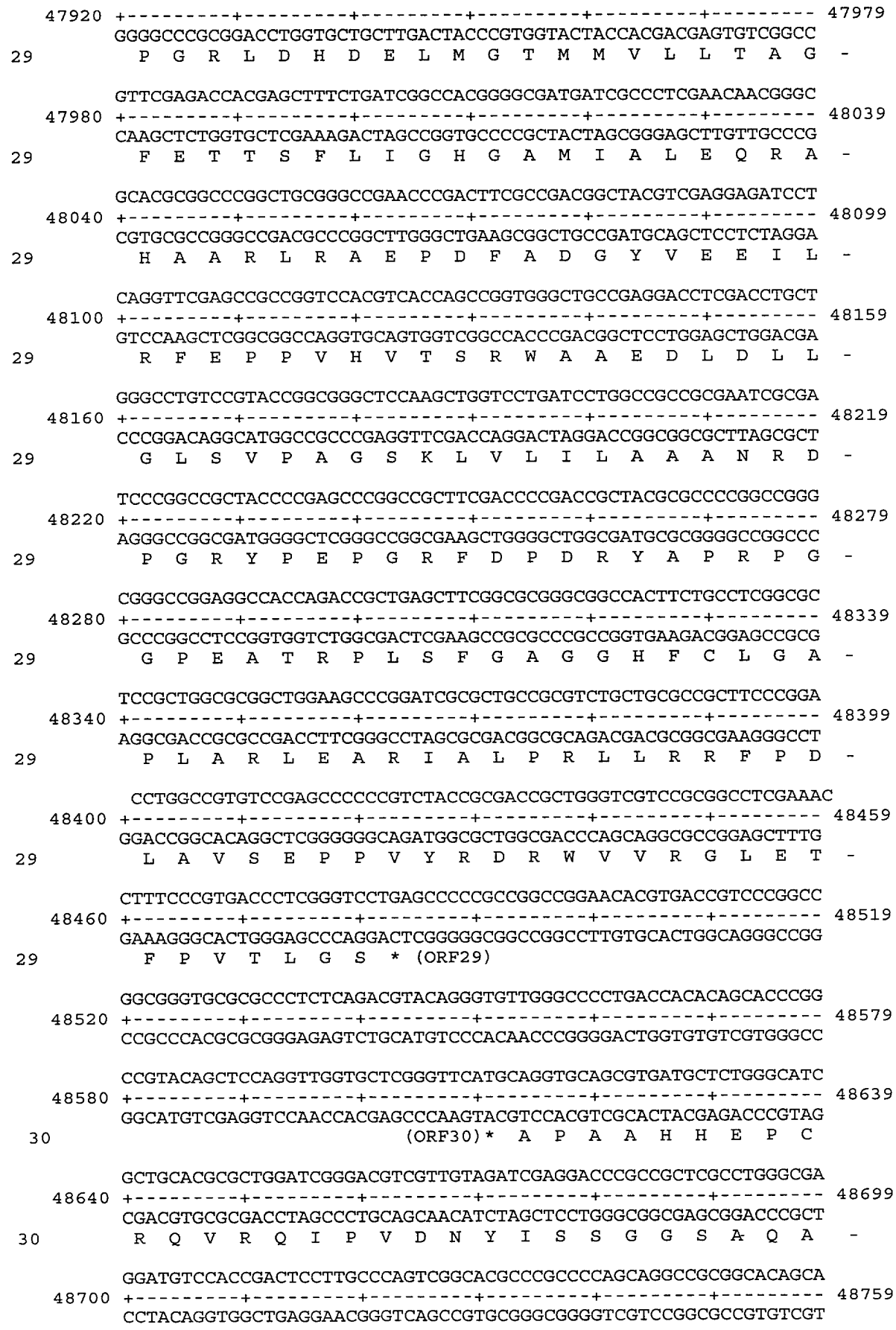

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30          V  R  E  E  L  T  W  A  E  G  S  A  G  K  S  D  V  L  D  A   -

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30          L  R  H  A  H  F  R  A  E  D  A  L  L  T  A  E  G  L  Q  L   -

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   48880    +---------+---------+---------+---------+---------+---------   48939
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30          H  T  I  P  A  S  G  Q  E  E  Y  E  T  Y  T  I  K  R  G  P   -

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   49240    +---------+---------+---------+---------+---------+---------   49299
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30          P  T  P  S  V  A  I  L  V  Q  W  H  S  H  W  A  G  S  I  F   -

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   49300    +---------+---------+---------+---------+---------+---------   49359
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30          G  W  K  G  N  V  V  V  G  G  D  V  P  A  A  M  G  G  P  S   -

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   49360    +---------+---------+---------+---------+---------+---------   49419
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30          L  T  G  S  V  R  V  D  P  R  S  F  V  E  D  Q  V  H  D  P   -

            AGAGGCCCGCCATCCAGGTGGGTATCCACCACACCGAGGCCGTCCAGGCGGCCGATCCGT
   49420    +---------+---------+---------+---------+---------+---------   49479
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30          F  L  G  A  M  W  T  P  I  W  W  V  S  A  T  W  A  A  S  G   -

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   49480    +---------+---------+---------+---------+---------+---------   49539
            GCGGCGCGCGGTCGAGCCGCCGGTGCAGGTGGTCCCACGCCCGCAGCCTGAGCTTCGGCG
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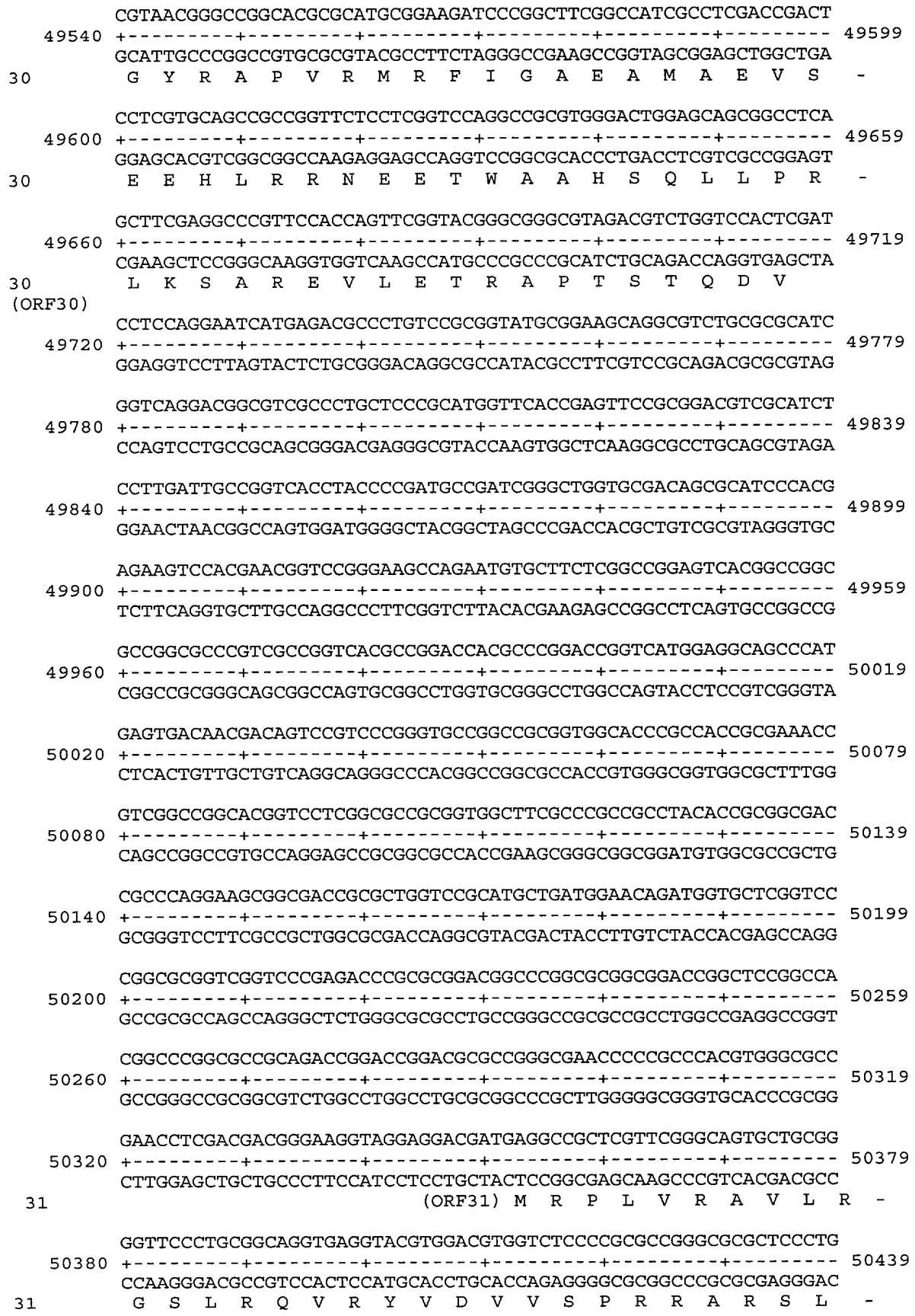

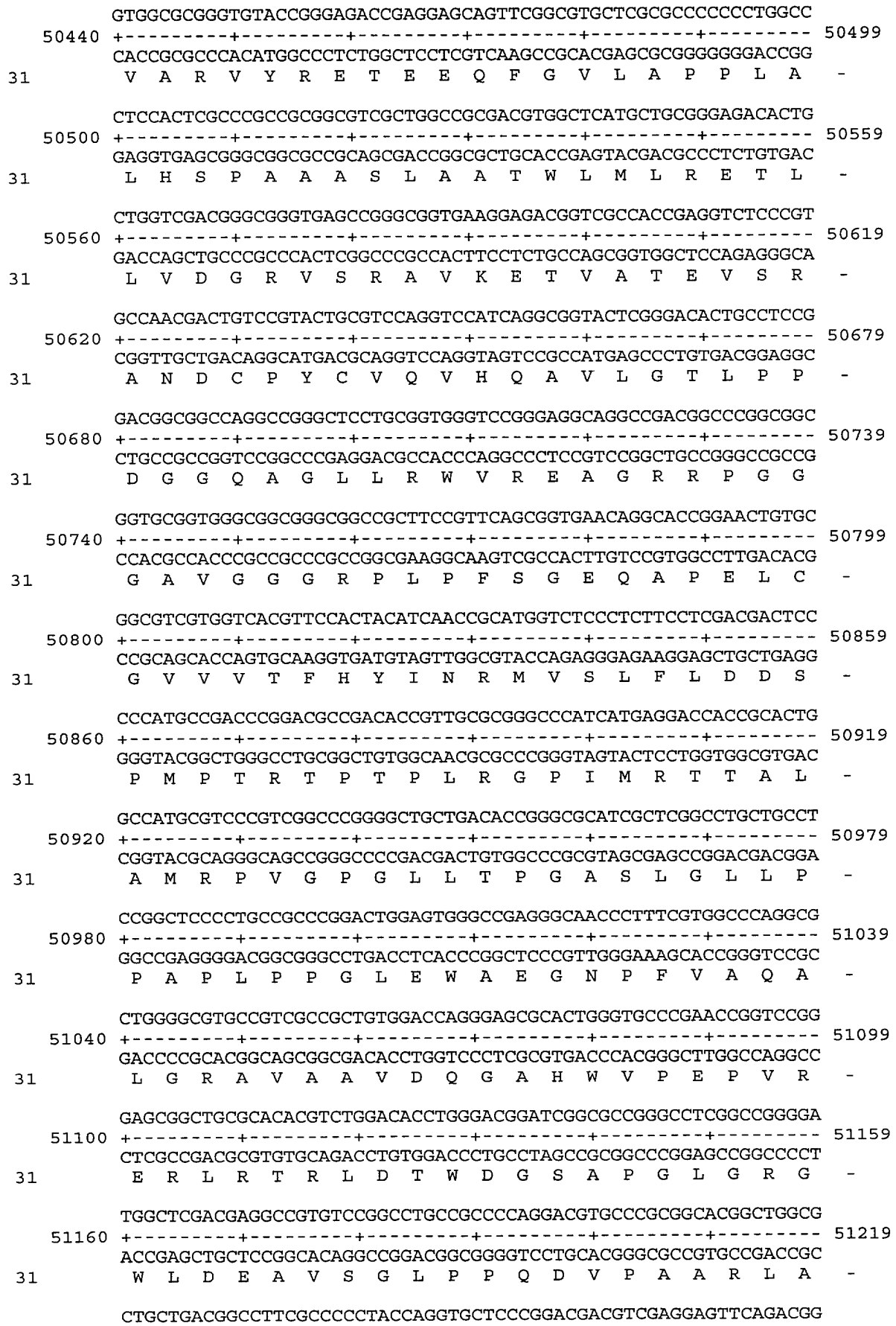

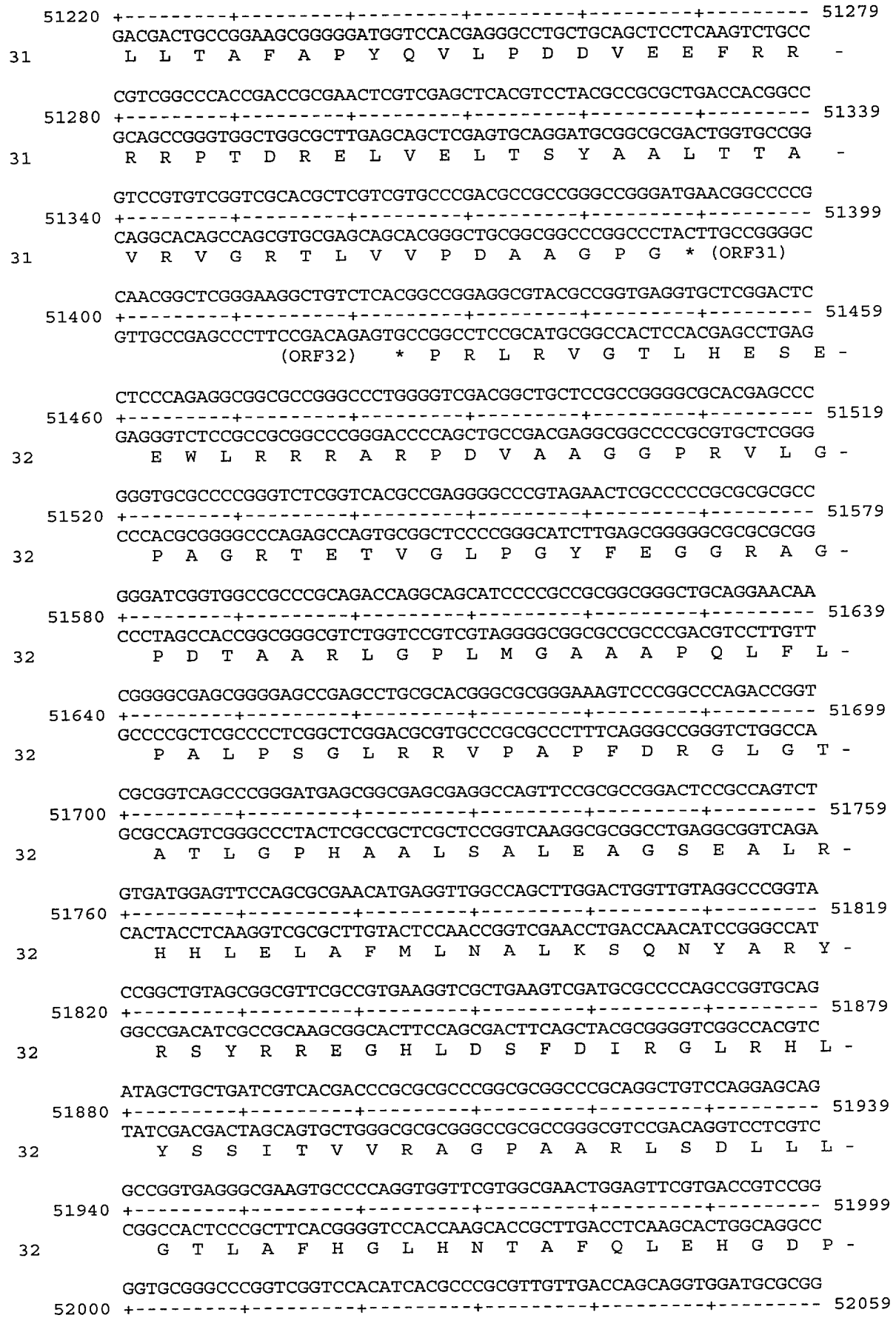
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51280 51339
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31 R R P T D R E L V E L T S Y A A L T T A -
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51340 51399
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31 V R V G R T L V V P D A A G P G * (ORF31)
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51400 51459
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51460 51519
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51520 51579
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51580 51639
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32 P D T A A R L G P L M G A A A P Q L F L -
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51640 51699
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32 P A L P S G L R R V P A P F D R G L G T -
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51760 51819
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51820 51879
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51880 51939
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51940 51999
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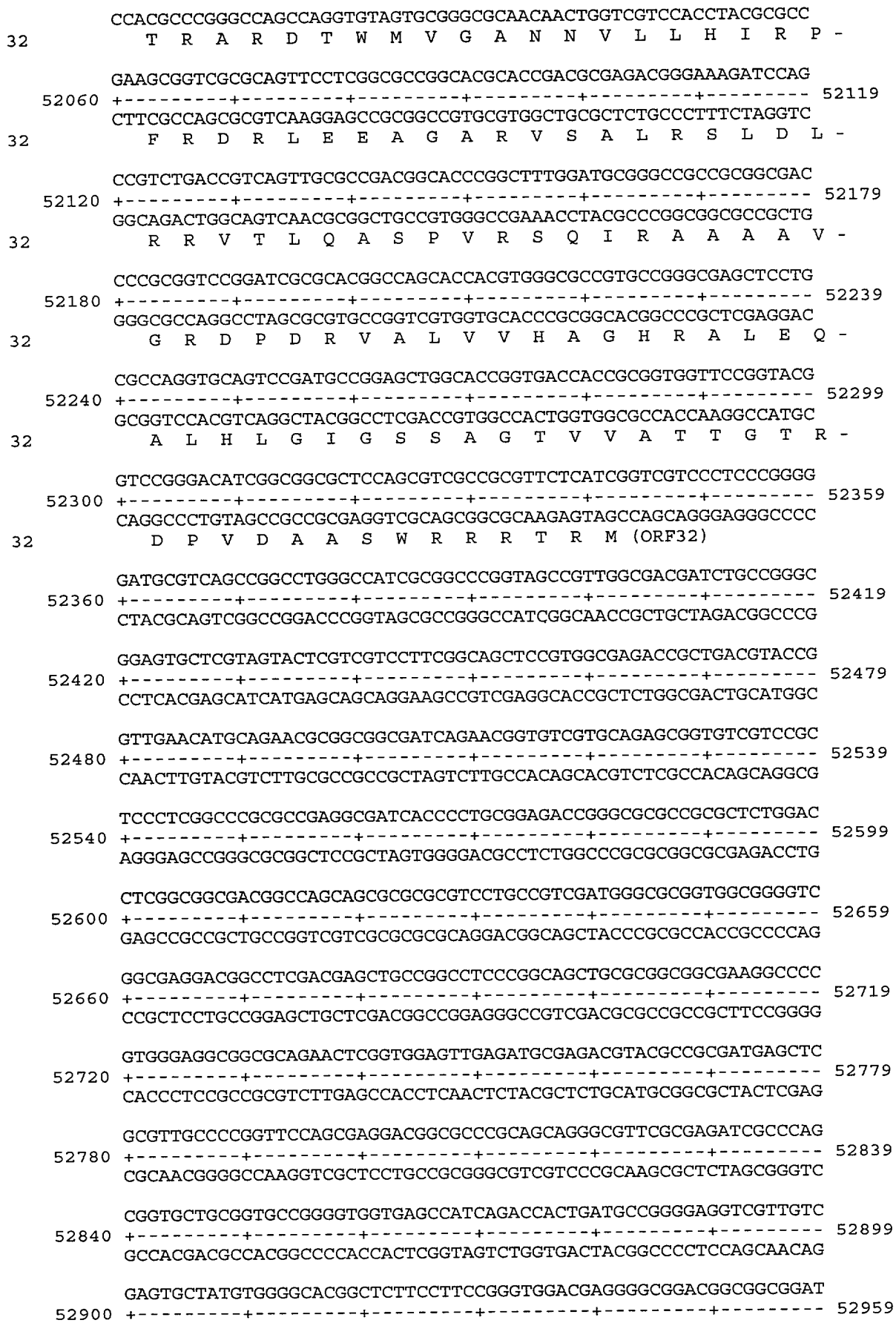

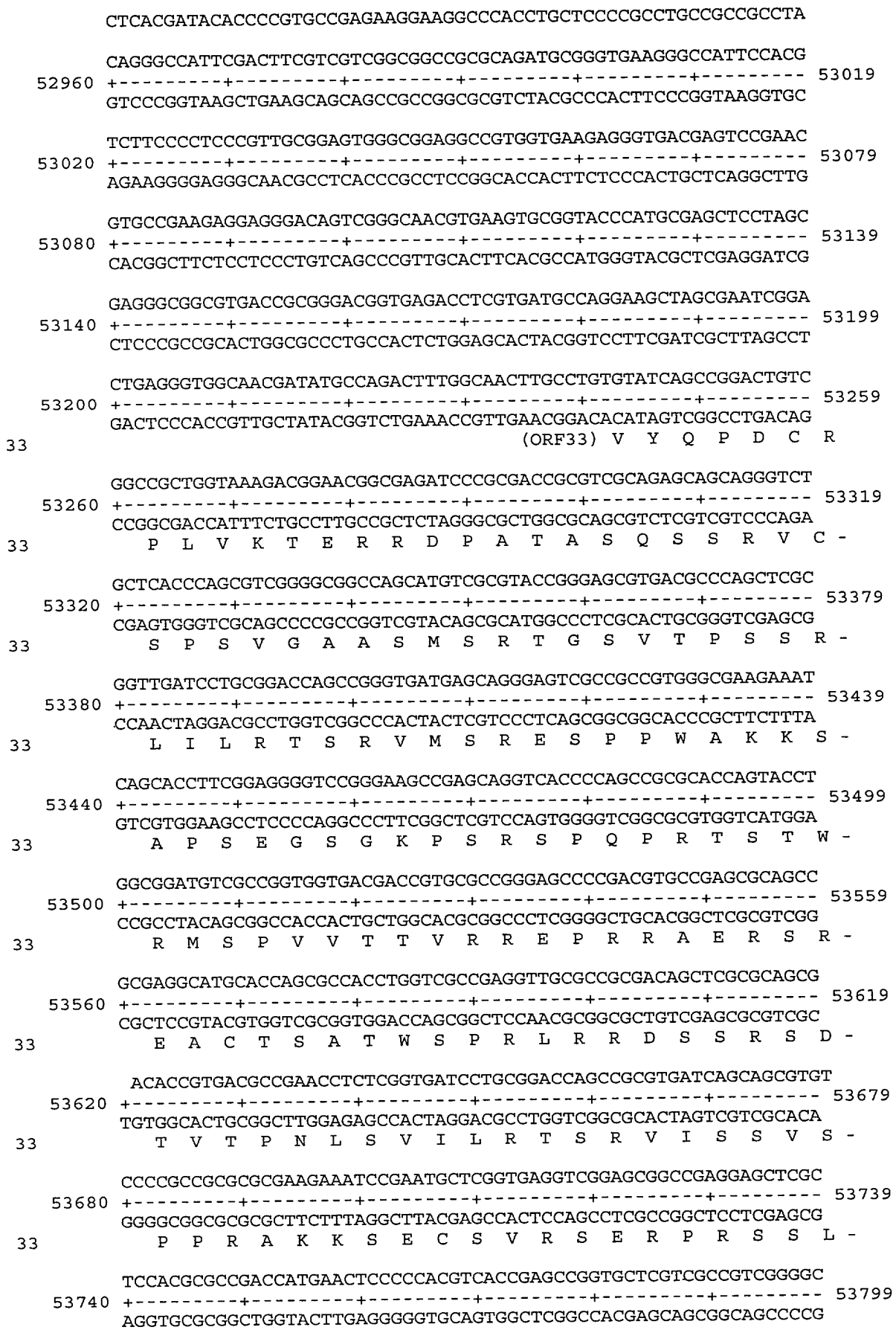

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   53800 +---------+---------+---------+---------+---------+--------- 53859
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33          F  G  A  P  D  P  A  E  R  F  R  P  E  T  A  E  R  S  L  V -

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   53860 +---------+---------+---------+---------+---------+--------- 53919
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33          T  F  A  T  S  R  G  M  C  R  L  H  R  L  P  A  T  V  R  E -

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   53920 +---------+---------+---------+---------+---------+--------- 53979
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33          H  M  L  H  G  N  T  F  P  S  R  W  Q  P  S  L  P  S  T  H -

        ACTGCAGTTGGGCGAGATGTGTAGGCATTCGAGGTCCGCAGGTTTGCCAAGCCGCGCGCG
   53980 +---------+---------+---------+---------+---------+--------- 54039
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33          C  S  W  A  R  C  V  G  I  R  G  P  Q  V  C  Q  A  A  R  D -

        ACCGGCATACTCTCTGGCACAACTGGAATGAGTAGCGTGGCAGGCCACGGGGACCGGGCC
   54040 +---------+---------+---------+---------+---------+--------- 54099
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33          R  H  T  L  W  H  N  W  N  E  *  (ORF33)

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   54100 +---------+---------+---------+---------+---------+--------- 54159
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   54160 +---------+---------+---------+---------+---------+--------- 54219
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        CGGGGGACGGCATGTCTACGGGCTATCTCTCGCGCCTGGAGTCGGGCGCCCGGCAGCCCT
   54220 +---------+---------+---------+---------+---------+--------- 54279
        GCCCCCTGCCGTACAGATGCCCGATAGAGAGCGCGGACCTCAGCCCGCGGGCCGTCGGGA
34          (ORF34)  M  S  T  G  Y  L  S  R  L  E  S  G  A  R  Q  P  S -

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   54280 +---------+---------+---------+---------+---------+--------- 54339
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34          D  R  A  V  A  H  L  A  G  Q  L  G  I  S  P  S  E  F  E  G -

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   54340 +---------+---------+---------+---------+---------+--------- 54399
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34          S  R  A  T  S  L  A  Q  I  L  S  L  S  T  S  L  E  S  D  E -

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   54400 +---------+---------+---------+---------+---------+--------- 54459
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34          T  S  E  L  L  A  E  A  V  R  S  A  H  G  Q  D  P  M  L  R -

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   54460 +---------+---------+---------+---------+---------+--------- 54519
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34          W  Q  A  L  W  L  L  G  Q  W  K  R  R  H  G  D  S  A  G  E -

        AGCACGGCTACCTCCAGCGTCTGGTGACGCTGAGTGAGGAGATCGGCCTGGCCGAGTTGC
   54520 +---------+---------+---------+---------+---------+--------- 54579
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34          H  G  Y  L  Q  R  L  V  T  L  S  E  E  I  G  L  A  E  L  R -

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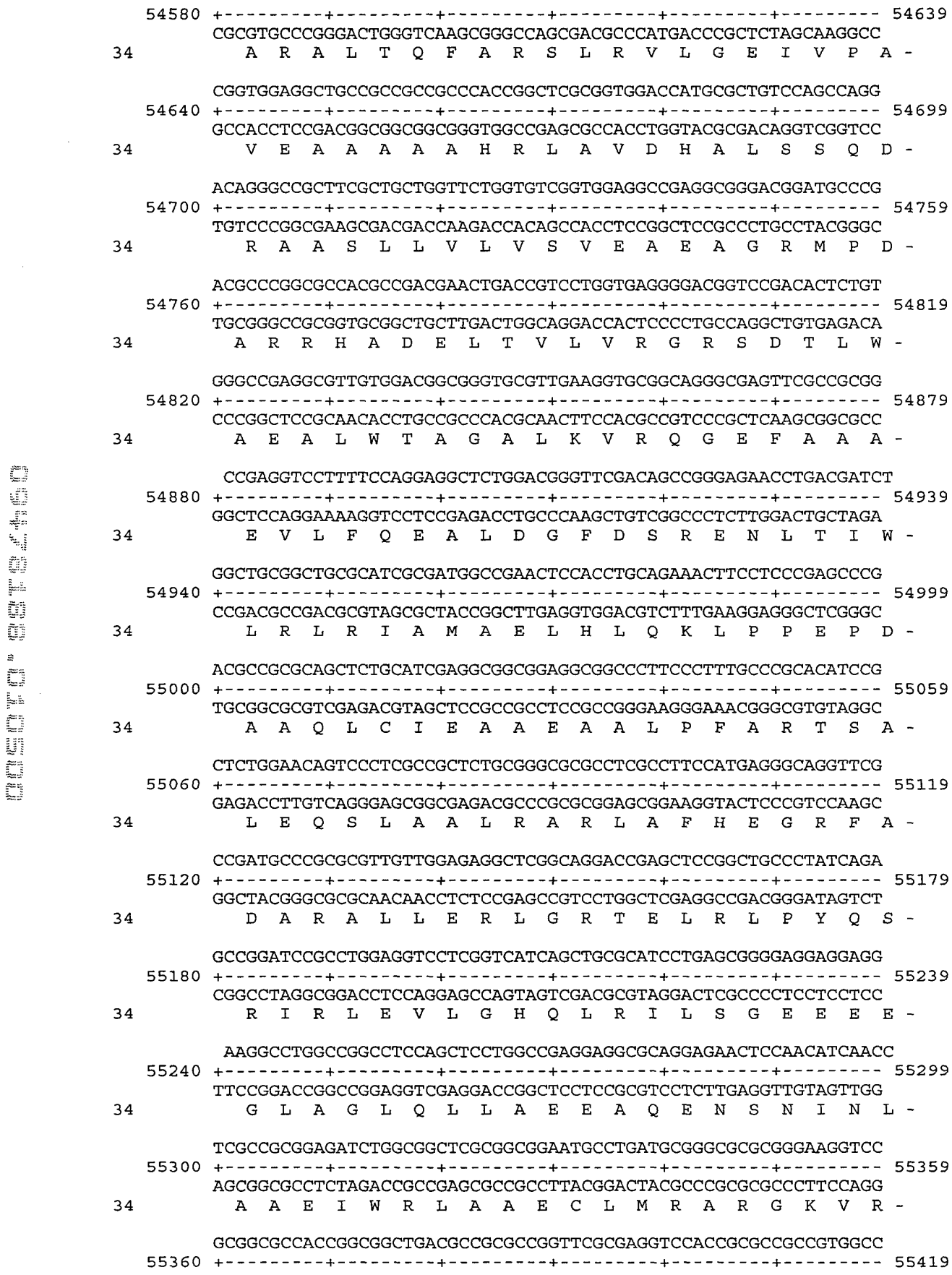

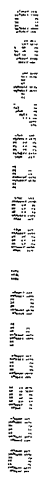

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34          G   A   T   G   G   * (ORF34)

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55420     +---------+---------+---------+---------+---------+---------+ 55479
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          TGCATCTGTTGGCACATGTGTACCTCCTACACAGTCAATTGTTGCCAAAATTGTCGAACC
55480     +---------+---------+---------+---------+---------+---------+ 55539
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55540     +---------+---------+---------+---------+---------+---------+ 55599
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55840     +---------+---------+---------+---------+---------+---------+ 55899
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55960     +---------+---------+---------+---------+---------+---------+ 56019
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56140     +---------+---------+---------+---------+---------+---------+ 56199
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56200     +---------+---------+---------+---------+---------+---------+ 56259
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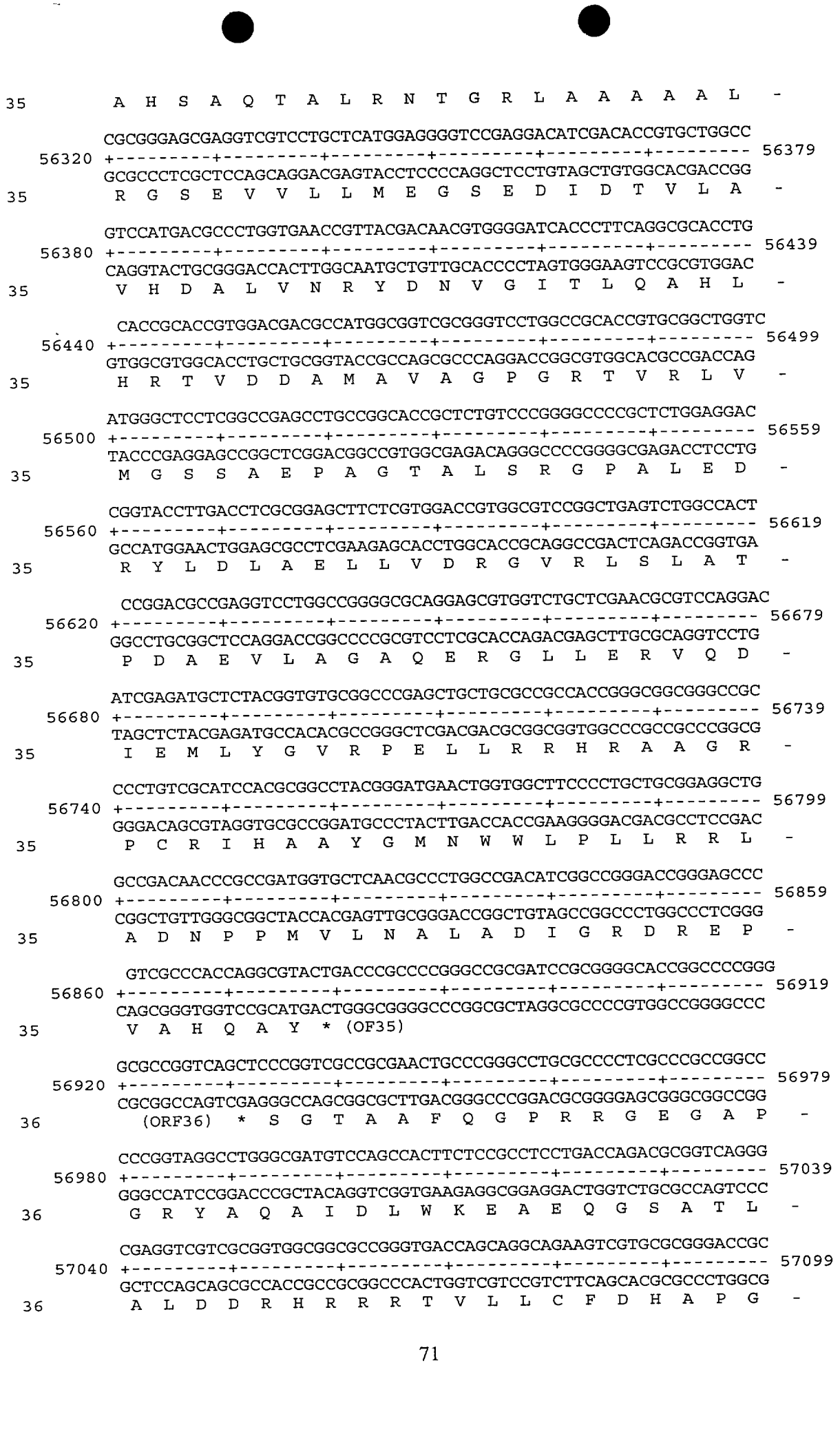
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56380 56439
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56440 56499
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35 M G S S A E P A G T A L S R G P A L E D -
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56560 56619
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56620 56679
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56680 56739
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56740 56799
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56800 56859
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56920 56979
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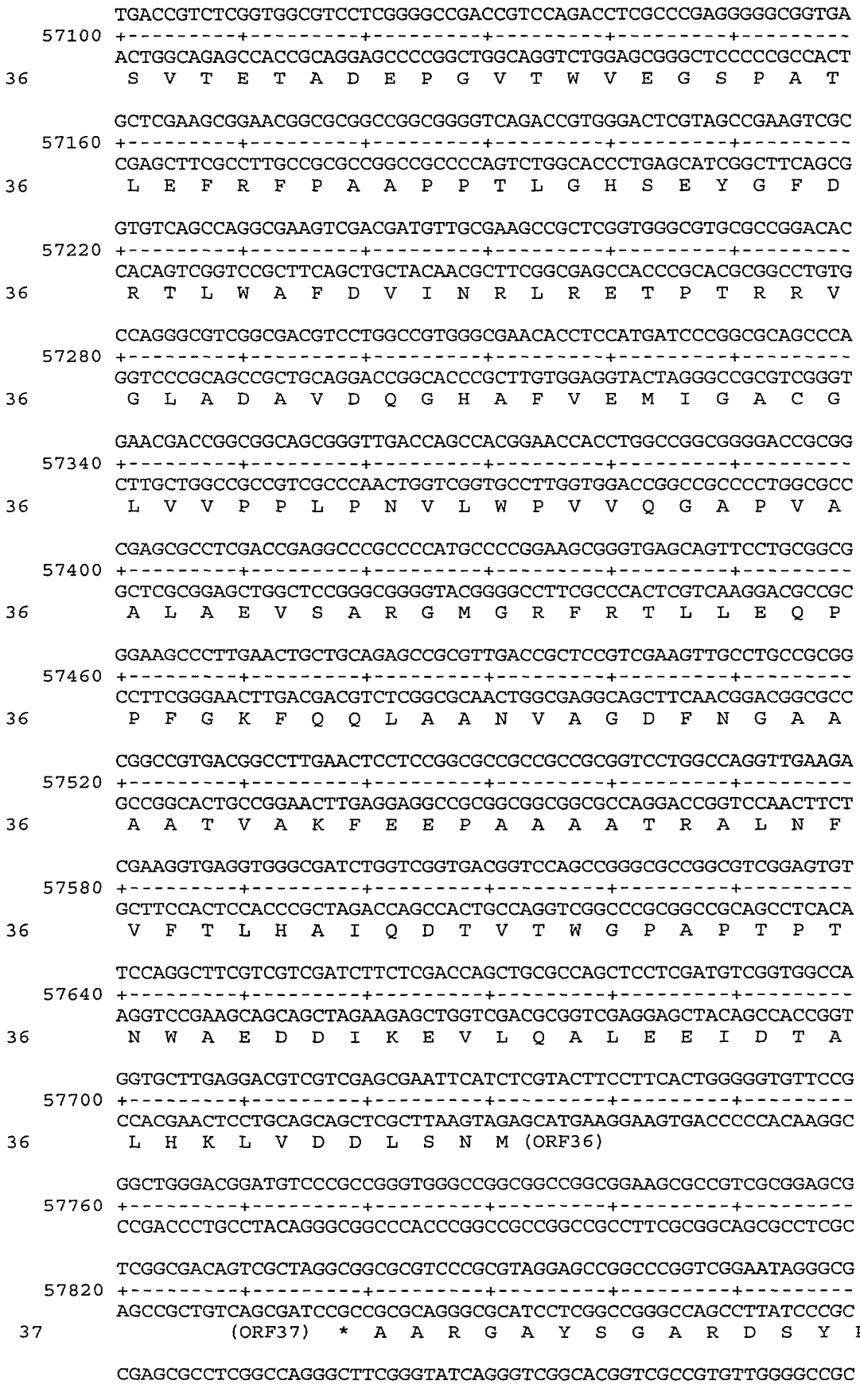

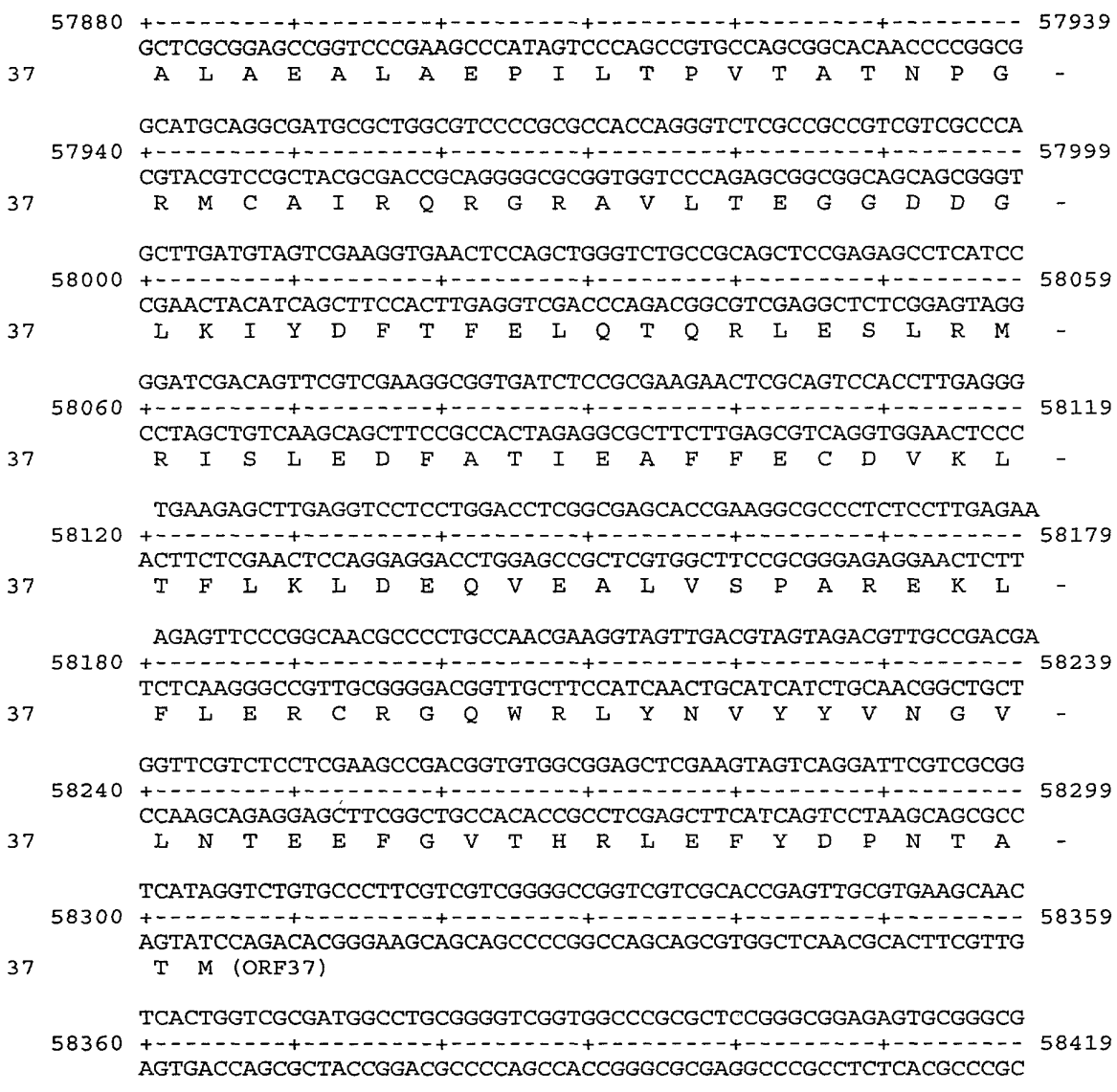

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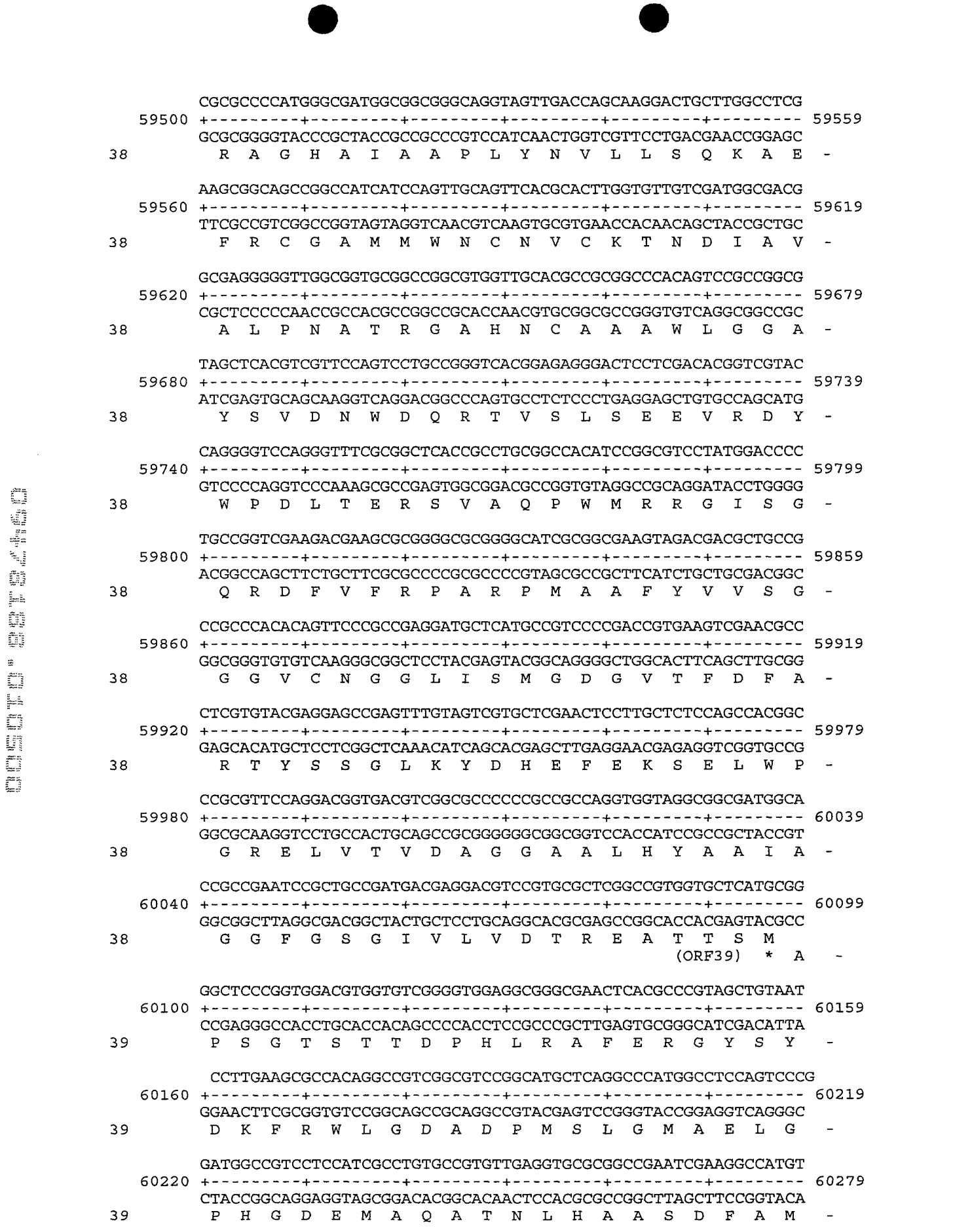

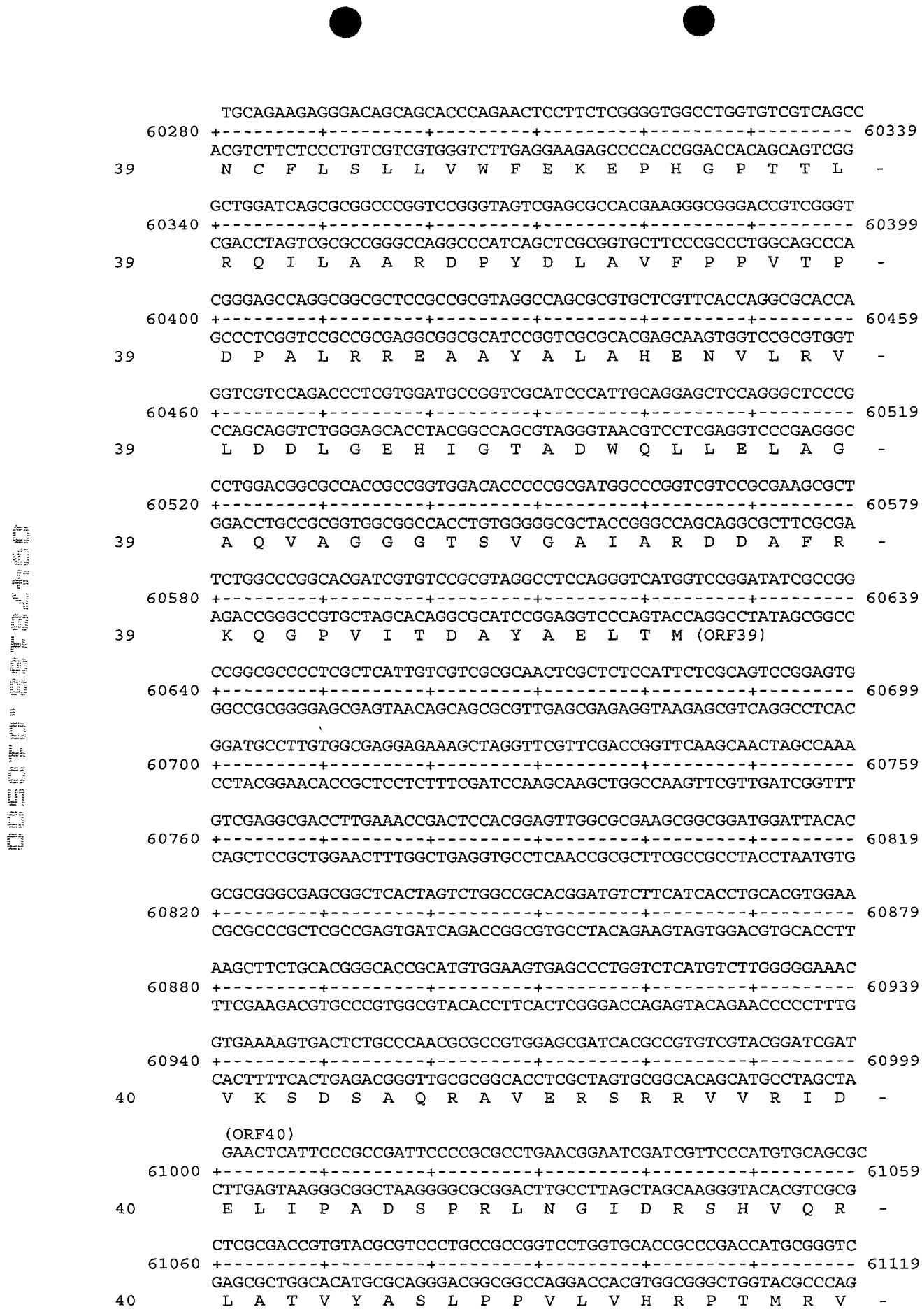

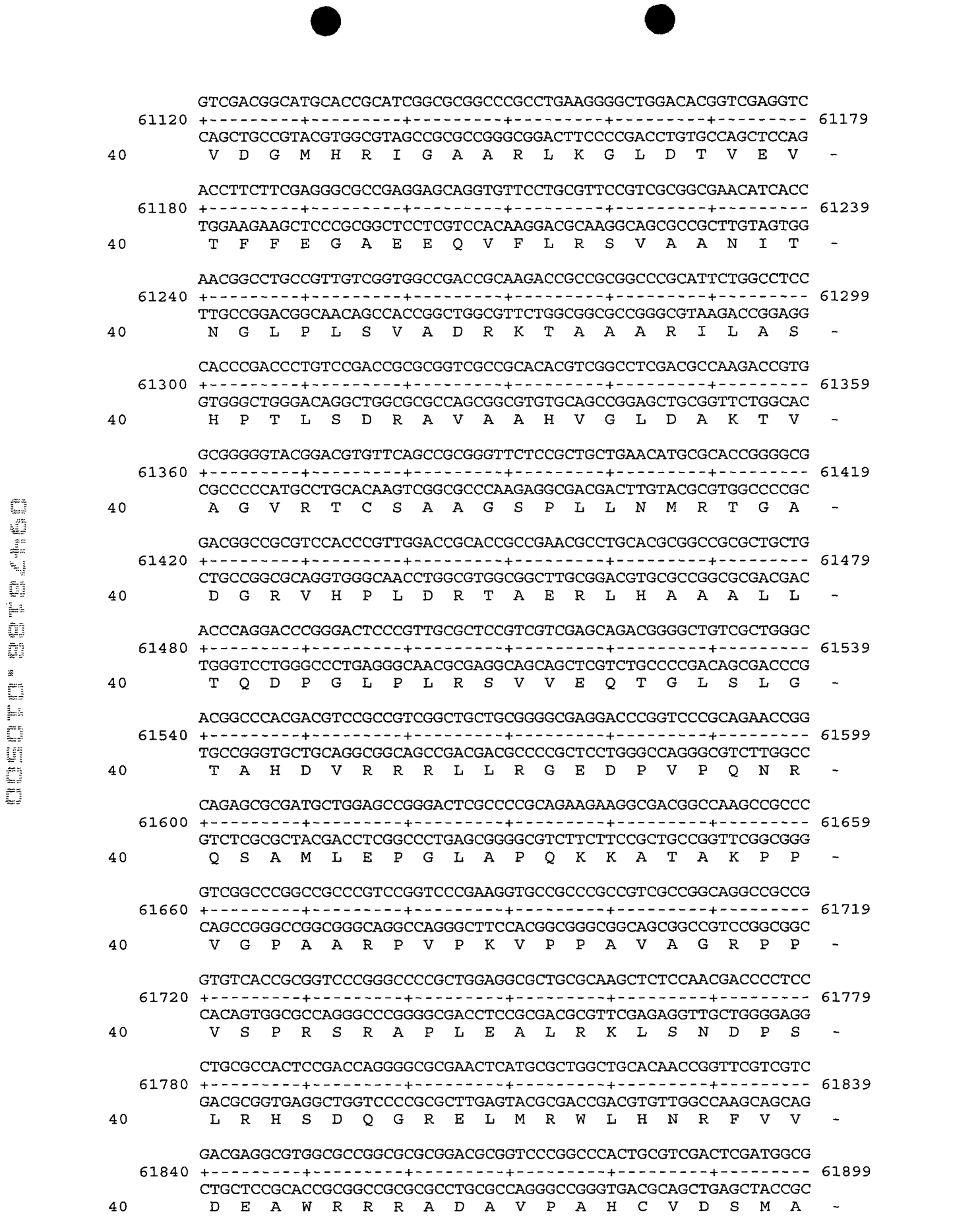
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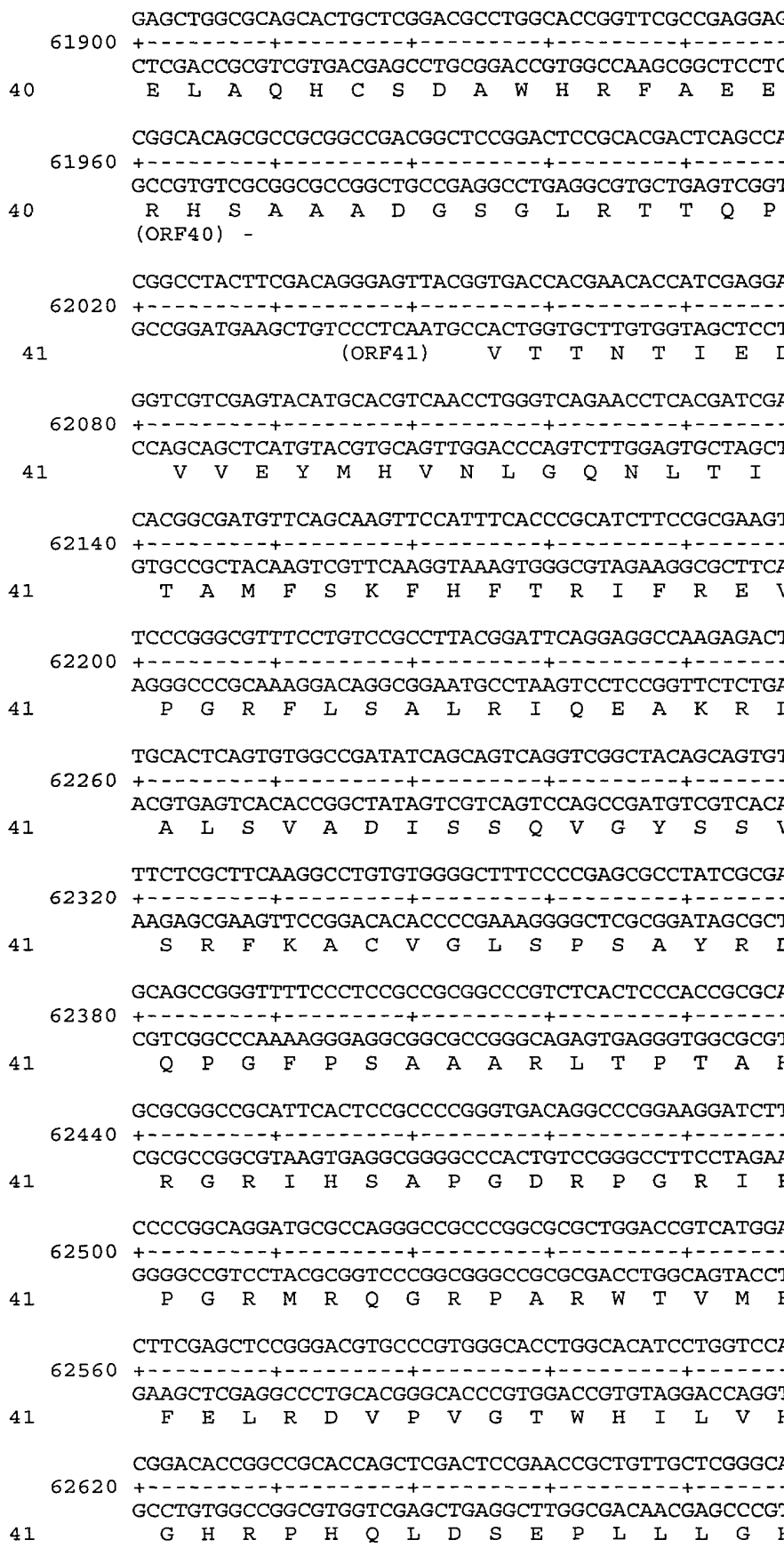
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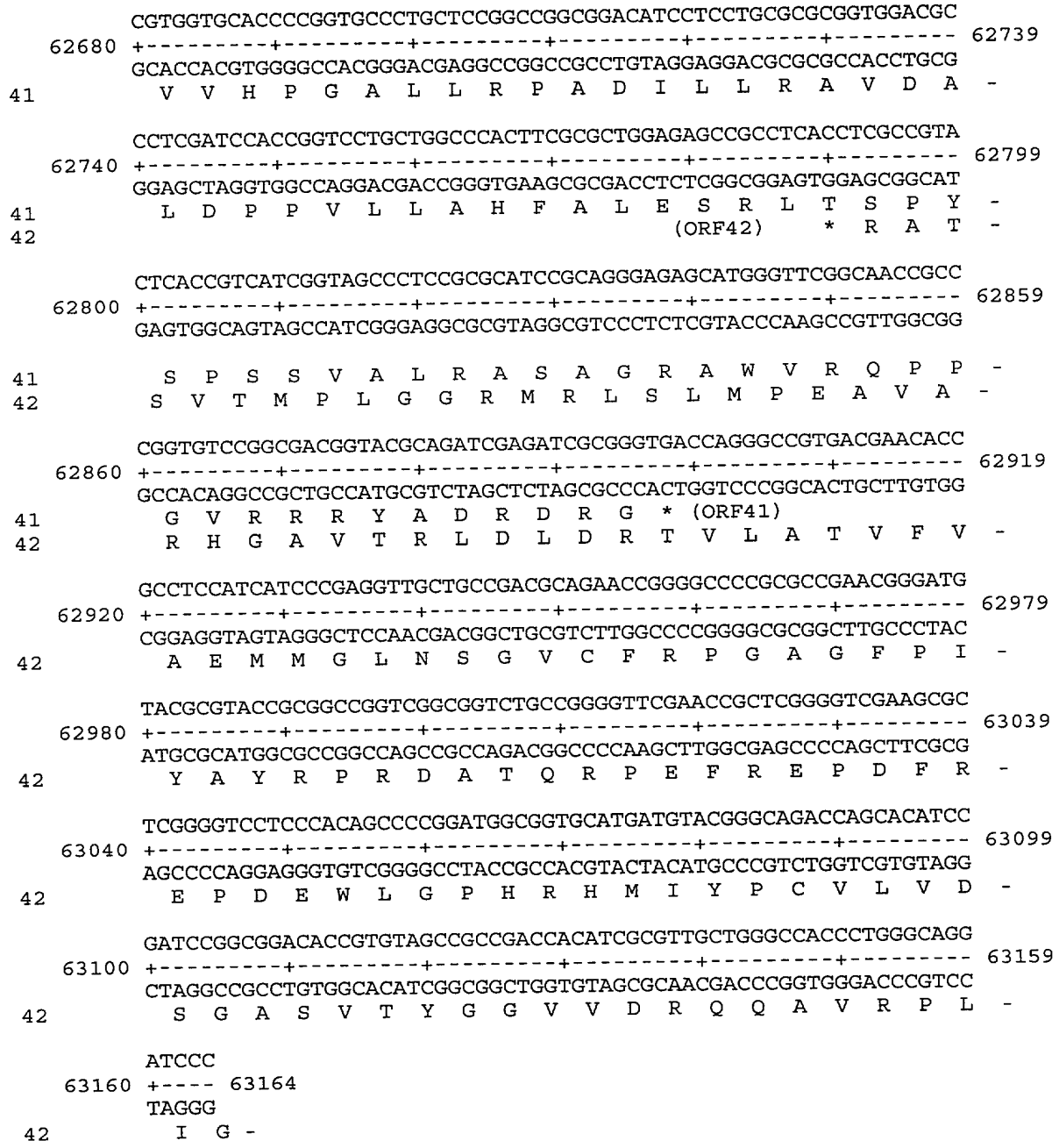
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42 Y A Y R P R D A T Q R P E F R E P D F R -
TCGGGGTCCTCCCACAGCCCCGGATGGCGGTGCATGATGTACGGGCAGACCAGCACATCC
63040 +---------+---------+---------+---------+---------+--------- 63099
AGCCCCAGGAGGGTGTCGGGGCCTACCGCCACGTACTACATGCCCGTCTGGTCGTGTAGG
42 E P D E W L G P H R H M I Y P C V L V D -
GATCCGGCGGACACCGTGTAGCCGCCGACCACATCGCGTTGCTGGGCCACCCTGGGCAGG
63100 +---------+---------+---------+---------+---------+--------- 63159
CTAGGCCGCCTGTGGCACATCGGCGGCTGGTGTAGCGCAACGACCCGGTGGGACCCGTCC
42 S G A S V T Y G G V V D R Q Q A V R P L -
ATCCC
63160 +---- 63164
TAGGG
42 I G -